Investigations into the Mechanisms that Are Responsible for Reduction in Colonic SN-38 Exposure

A Dissertation Presented to the

Faculty of the Department of Pharmacological and Pharmaceutical Sciences

College of Pharmacy, University of Houston

In Partial Fulfillment of

the Requirements for the Degree of

Doctor of Philosophy

By

Sumit Basu

July, 2015

ACKNOWLEDGEMENTS

At the end of this memorable journey in University of Houston I would like to thank those who made the journey deeply enjoyable and productive. I have learnt so many things- both technical and nontechnical - from them. Here the small scope would let me mention only some of them.

To begin with, I express my extreme indebtness to my advisor, Dr. Ming Hu, for his guidance and inspiration throughout these years. His advices were indispensable in my research. The support I got from him in critical situations is unforgettable. To sum up, it has been a lifetime experience for me to work under his supervision, and this will always lead me to be a good researcher and a good human being as well.

I would also like to sincerely thank my dissertation committee members, Drs. Diana Chow, Vincent Tam, Tim Cooper and Michael Overman for their continuous supports, invaluable suggestions and comments regarding my work on several occasions. I am obliged to Dr. Brian Knoll, Dr. Tahir Hussain, Dr. Douglas Eikenburg and Dr. Romi Ghose for their help in different stages of my stay here.

I have to also thank Dr. Song Gao and Ms. Taijun Yin from University of Houston, and Dr. Min Zeng and Mr. Rongjin Sun from Hubei. Without their technical supports it would be impossible for me to complete this thesis work.

Special thanks go to all my PPS colleagues and labmates in University of Houston. I am glad to have friends like Santosh, Tanay, Sonal, Pranav, Zhiyi, Pankajini, Nashid,

Manas, Prajakta, Mahua, Yong, Yu, Vishal and Tao. They made my stay in Houston both inspiring and enjoyable.

I also want to thank fellow senior graduate students, especially Dr. Kaustubh Kukarni, Dr. Baojian Wu, Dr. Adarsh Gandhi and Dr. Kaustuv Saha, of the Department of Pharmacological and Pharmaceutical Sciences for all the supports you gave me.

I wish to thank my teachers and friends from my alma maters, Jadavpur University.

Lastly, but not the least, special thanks to Arijita and my family members who are always being there, encouraging and inspiring me in all my pursuit. Thank you for all your unconditional love and support.

Sumit Basu

Abstract

Statement of the Problem: Irinotecan is used as a single agent or in combination with other drugs to treat metastatic colorectal cancer. However, its usage is largely limited as it exhibits late onset diarrhea in 30-40% of the patient population due to the high accumulation of SN-38 in colon. It has been hypothesized that multiple factors, such as higher biliary excretion of SN-38 and SN-38 glucuronide mediated through different efflux transporters, deglucuronidation of SN-38 glucuronide to SN-38 by bacterial β-glucuronidase secreted by commensal microbiota, generation of toxic active metabolite SN-38 from irinotecan mediated by intestinal carboxylesterse enzymes in gut and enterohepatic recycling of irinotecan and SN-38, etc., can contribute to the elevation of colonic SN-38 content resulting in severe late onset diarrhea. In this particular study, we aimed to investigate the mechanisms that are responsible to reduce the colonic content of SN-38. Towards our goal, three major specific aims were proposed to: (1) develop a modified *in situ* rat perfusion model for determination of the disposition of irinotecan, SN-38 and SN-38 glucuronide in major metabolic organs and bio-matrices; (2) investigate the potential of certain chemicals and natural products to reduce colonic exposure of SN-38 by inhibiting biliary and intestinal excretions of SN-38 and SN-38 glucuronide employing the modified *in situ* rat perfusion model; and (3) determine if the selected chemicals and natural products can decrease the colonic exposure of SN-38 by decreasing fecal content of SN-38 employing *in vivo* pharmacokinetic study.

Methods: Surgeries and perfusion experiments were carried out on three different groups of wistar rats (n=4) at different dose of intravenous irinotecan (0.5, 5 and 50

mg/kg) for 150 minutes. Bile, perfusate, urine and plasma were collected at each 30 min interval. Irinotecan, SN-38 and SN-38 glucuronide concentrations from all the collected bio-matrices were quantified by our developed and validated LC-MS/MS assay. Chemical inhibition study using different chemical inhibitors of efflux transporters have been performed to explore the impacts on efflux transporters for the disposition of irinotecan. Traditional Chinese herbal formulations have been coadministered with irinotecan to observe if they can alter the disposition of irinotecan significantly. Lastly, *in vivo* rat pharmacokinetic study was conducted to evaluate the effectiveness of chemical inhibitor and Chinese herbal formulation to decrease the fecal content of SN-38 without altering the efficacy of chemotherapeutic treatment.

Results: Dose response study using the modified *in situ* rat perfusion model revealed that predominant excretion of irinotecan is biliary route (45 to 60%) in comparison to urinary (4 to 15%) and intestinal excretion (3 to 10%). In addition, the high steady state concentrations of irinotecan and its metabolites in bile and urine compared to their plasma concentrations (5 to 5000 fold) displayed that the excretion of these compounds at different metabolic organs was mainly governed by predominant efflux transporters. Chemical inhibition study using the same perfusion model demonstrated the possible involvement of MATE-1 transporter alongwith P-gp and MRP2 on the biliary and renal excretions of irinotecan. Inhibition study using Chinese herbal medicine indicated that Xiao Chao Hu Tang (XCHT) could decrease the biliary and intestinal excretions of SN-38 and SN-38 glucuronide by 60 -90%. The *in vivo* rat pharmacokinetic study using cimetidine and XCHT revealed that both XCHT and cimetidine could decrease the fecal

content of SN-38 by 40-50% without altering the efficacy of the chemotherapeutic treatment.

Conclusions: In this particular study, we demonstrated that biliary route is the predominant excretion route for irinotecan, SN-38 and SN-38 glucuronide compared to other excretion routes. In addition, effluxes of irinotecan and its metabolites in biliary and urinary routes, respectively, are mainly governed by efflux transporters. Chemical inhibition study utilizing the modified *in situ* rat perfusion model demonstrated the possible involvement of MATE-1 transporter on the disposition of irinotecan through biliary and urinary routes. We have also demonstrated using the modified perfusion model as a screening tool that Chinese herbal medicine Xiao Chao Hu Tang (XCHT) has the ability to decrease the biliary and intestinal excretions of SN-38 and SN-38 glucuronide. Lastly, to the best of our knowledge, this is the first time cimetidine and Chinese herbal formulation XCHT have displayed their abilities to decrease fecal excretion of SN-38 which may lead to amelioration of irinotecan induced late onset diarrhea. However, further investigations are warranted to prove their effectiveness in alleviating irinotecan toxicity using a preclinical diarrhea model.

List of Tables

List of Figures

List of Abbreviations

BCRP	Breast Cancer Resistant Protein
CAM	Complementary and Alternative Medicine
CES/CE	Carboxylesterase
CL_{int}	Intrinsic clearance
CPT	Camptothecin
CYPs	Cytochrome P450
DDB	Drug Detoxifying Bacteria
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
G-CSF	Granulocyte Colony Stimulating Factor
K_m	Michaelis constant
MATE-1	Multidrug Anionic Toxic Extrusion-1
MRP2	Multidrug Resistant Protein-2
MS	Mass spectroscopy
P-gp	P-glycoprotein
SN-38	7-Ethyl-10-hydroxycamptothecin
SST	Sho-Saiko-To
TCM	Traditional Chinese medicine
UDPGA	Uridine diphosphoglucuronic acid
UGT	UDP-glucuronosyltransferase
UGT71G1	Triterpene/flavonoid glycosyltransferase from the legume *Medicago truncatula*
UPLC	Ultra performance liquid chromatography
V_{max}	Maximum velocity
XCHT	Xiao Chao Hu Tang

Contents

Chapter 1 Review of the literature

1.1. Introduction to the project

Irinotecan (CPT-11) is a semisynthetic derivative of camptothecin (CPT) extracted from the bark of *camptothecin acuminate* (Wall M. E. 1966; Fukuoka, Niitani et al. 1992). It is extensively used as a firstline therapy against metastatic colorectal cancer as a single agent or in combination with other anticancer drugs like 5-flurouracil, oxaliplatin etc. (Tournigand, Andre et al. 2004; Akhtar, Chandel et al. 2014). Apart from colorectal cancer, irinotecan also demonstrated promising clinical activity against other types of cancer such as gastric, pancreatic, ovarian, lung (both small and non small cell), brain etc. (Ohno, Okada et al. 1990; Fukuoka, Niitani et al. 1992). The clinical usage of irinotecan is often restricted owing to its severe side effects namely, neutropenia and late onset diarrhea. Among these two side effects, neutropenia is more common and can be treated permanently by granulocyte colony stimulating factor (G-CSF) (Hecht, Pillai et al. 2010). On the other hand, late onset diarrhea is regarded as a life threatening side effect which is observed in approximately 30 - 40% of the patient population especially at higher dose of irinotecan (Rosen 1998; Rothenberg, Meropol et al. 2001; Hecht, Pillai et al. 2010).

After intravenous administration, irinotecan forms its active metabolite SN-38 through the action of liver carboxylesterse (CES) enzymes. Like all other camptothecin derivatives, both irinotecan and SN-38 also demonstrated their cytotoxic action after forming an irreversible complex with DNA topoisomerase I enzyme which is crucial for regulation of DNA replication (Redinbo, Stewart et al. 1998; Stewart, Redinbo et al. 1998). In terms of

cytotoxicity, SN-38 is 100-1000 times more potent than irinotecan (Mathijssen, van Alphen et al. 2001). But owing to the poor solubility of SN-38, irinotecan is used as a prodrug to treat colorectal cancer. Usually SN-38 undergoes glucuronidation by phase II metabolizing enzyme uridine 5'-diphospho-glucuronosyltransferase enzymes (UGT) to form inactive and non-toxic SN-38 glucuronide which can also reverse back to SN-38 in intestine by the deconjugation reaction carried out by the bacterial β glucuronidase enzyme secreted from commensal microbiota (Tukey and Strassburg 2000; Mathijssen, van Alphen et al. 2001).

Mechanistic studies indicated that direct action of SN-38 towards the intestinal epithelum causes late onset diarrhea (Hecht 1998). It has been hypothesized that multiple factors which can elevate the accumulation of SN-38 in colon can ultimately lead to late onset diarrhea. Regeneration of SN-38 from SN-38 glucuronide by bacterial β glucuronidase secreted from commensal microbiota in the gut, direct activation of SN-38 from prodrug irinotecan in intestine and excessive biliary and intestinal secretion of SN-38 by different efflux transporters etc. can directly increase SN-38 content in colon which results in severe diarrhea (Catimel, Chabot et al. 1995; Ahmed, Vyas et al. 1999; Kehrer, Sparreboom et al. 2001).

Till date, several approaches have been taken to reduce the severity of late onset diarrhea induced by irinotecan; but none of them was completely successful to alleviate the incidence of diarrhea. In this particular project, we investigated different mechanisms which may reduce SN-38 content in colon leading to possible amelioration of irinotecan induced diarrhea. At first, we developed and validated a UPLC-MS/MS method to

determine irinotecan, SN-38 and SN-38 glucuronide simultaneously in different bio-matrices (bile, plasma, perfusate and urine) and major metabolic organs (liver and kidney). Then we conducted a dose response study of irinotecan (0.5, 5 and 50 mg/kg) utilizing a modified *in situ* rat perfusion model which demonstrated the pattern of distribution and elimination of irinotecan and its metabolites in different bio-matrices as well as in metabolic organs. Next, we explored the impact of efflux transporters on the disposition of irinotecan and its metabolites at different organs as well as screened some chemical inhibitors and traditional herbal formulations which can decrease the biliary and intestinal excretions of SN-38 and SN-38 glucuronide by employing the developed modified perfusion model. Lastly, we performed *in vivo* pharmacokinetic study to evaluate the ability of selected chemical inhibitor and herbal formulation to decrease the fecal excretion of SN-38 which may reduce the incidence of late onset diarrhea.

The thesis is divided into six chapters. In chapter 1, detailed background with literature review as well as introduction for the proposed study is included. Chapter 2 discusses the statement of purpose, specific aims and general strategy of the project. From chapter 3 through chapter 5, the proposed studies are discussed in detail alongwith the research findings. In chapter 3, we have discussed how a UPLC-MS/MS method of irinotecan and its metabolites have been developed and validated in different bio-matrices and major metabolic organs with its application in pharmacokinetic studies. Chapter 4 discusses about the development of a modified *in situ* rat perfusion model which demonstrated the disposition of irinotecan in different bio-matrices and major metabolic organs. In addition, we also explored the impact of predominant efflux

transporters on the disposition of irinotecan in different metabolic organs in chapter 4. In chapter 5, we investigated the capability of herbal formulations to alter the disposition pathways of irinotecan and also conducted the pharmacokinetic studies to observe if our selected chemical inhibitor and herbal formulation can decrease the fecal content of SN-38 without altering the efficacy of the chemotherapeutic treatment. Then in chapter 6 we summarized all of the key findings of this project. Lastly, we added the preliminary results of another approach to reduce colonic SN-38 content termed as “Drug detoxifying bacteria” in the appendix section.

1.2. Primary use of irinotecan

At present irinotecan is mainly used as a first line drug to treat metastatic colorectal carcinoma (Akhtar, Chandel et al. 2014). It is basically used as an important component of a regimen FOLFIRI which includes other chemotherapeutic drug such as 5-fluroouracil and leucovorin (Tournigand, Andre et al. 2004; Akhtar, Chandel et al. 2014). It received full FDA approval to treat metastatic colorectal carcinoma on 1998 (Cunningham, Pyrhonen et al. 1998; Saltz, Cox et al. 2000). Irinotecan is used as a single agent regimen at the dose of 125 to 350 mg/m^2 in clinic. Usually, 125 mg/m^2 dose of irinotecan is infused over 90 min weekly, whereas 350 mg/m^2 dose is administered every three weeks (Rothenberg, Kuhn et al. 1998). As irinotecan exhibited severe toxicity like neutropenia and diarrhea, so dose modification of irinotecan as well as delay in chemotherapeutic treatment until the recovery of patient is often observed. Apart from metastatic colorectal cancer, irinotecan also exhibited promising clinical activity against different types of cancer such as lung (both small and non-small), gastric, ovarian, brain,

pancreatic, cervical, leukemia, lymphoma etc. (Ohno, Okada et al. 1990; Fukuoka, Niitani et al. 1992; Rosen 1998).

1.3. Mechanism of Action of irinotecan

Generally, camptothecins (CPT) exhibit the anticancer action by trapping the topoisomerase I enzyme which causes an unusual damage to the DNA (Hsiang, Hertzberg et al. 1985; Redinbo, Stewart et al. 1998; Stewart, Redinbo et al. 1998). Generally, toposiomerase I enzyme creates reversible single strand breaks in DNA after cutting and reattaching the double chain of DNA which relieve the torsional strain produced by the replication forks culminating in DNA replication process (Shao, Cao et al. 1999). Both irinotecan and SN-38 binds to the topoisomerase I-DNA complex and causes cell death by disrupting the double strand DNA.

1.4. Toxicity of irinotecan

Though irinotecan is considered as an important component of the firstline therapeutic regimen against metastatic colorectal cancer, severe toxicity of irinotecan has limited its usage by a considerable extent. Generally irinotecan imparts severe dose limiting toxicities like myelosuppresion, neutropenia, and most prominent one, diarrhea which are caused due to its complicated interaction with drug metabolizing enzymes and efflux transporters (Mathijssen, van Alphen et al. 2001). Among all the drug metabolizing enzymes, UGT1A1 plays the most significant role in the irinotecan induced toxicity as it actively participates in the detoxification of active metabolite SN-38 to non-toxic SN-38 glucuronide (Tukey and Strassburg 2000). Pharmacogenetic studies indicated that

variation of UGT1A1 gene causes alteration of glucuronidation process of SN-38 ultimately resulting in the occurrence of neutropenia and diarrhea (Tukey and Strassburg 2000; Mathijssen, van Alphen et al. 2001).

It is estimated that 70% of patients receiving irinotecan treatment suffer from diarrhea, which makes an increased dosing schedule difficult. Approximately 55% of these patients receive a lower dose than scheduled after 1 month (Brandi, Dabard et al. 2006). The diarrhea associated with irinotecan usage is of two types, early onset and late onset. Usually early onset diarrhea starts within 24 hours after administration of irinotecan. It is mild and transient in nature. Generally it is assumed that anti-cholinesterase activity of irinotecan promotes the early onset diarrhea which destroys the secretory and absorptive functions and properties of the intestinal mucosa. In addition, SN-38 possess mitotic inhibitory properties which also contribute to the structural and functional defects in the intestinal lumen (Takasuna, Hagiwara et al. 1996). Most of the symptoms of early onset diarrhea are like cholinergic syndrome which includes salivation, visual disturbances, gastrointestinal cramps, lacrimation etc. Anticholinergic drug atropine (0.25 – 1 mg i.v.) is the firstline treatment of treating early onset diarrhea (Gandia, Abigerges et al. 1993; Bleiberg and Cvitkovic 1996; Rougier, Bugat et al. 1997). Alternatively, antihistaminic drugs (diphenhydramine), serotonin 5-HT_3 antagonist (ondansetron), antimuscuranic drug (scopolamine) etc. can be used as premedication therapy to alleviate the symptoms (Rowinsky, Grochow et al. 1994; Zampa, Magnolfi et al. 2000).

Late onset diarrhea caused by irinotecan begins more than 24 hours of infusion (Rougier, Bugat et al. 1997). The nature of the late onset diarrhea is dose and schedule dependent, inconsistent and unpredictable (Abigerges, Chabot et al. 1995; Hecht 1998). In addition, it varies widely patient to patient. Prolong duration of late onset diarrhea can lead to dehydration as well as imbalance of electrolytes which consequently worsens patient's condition to life threatning. Although till date there is no exact mechanism which can explain irinotecan induced late onset diarrhea, it is postulated that direct intestinal mucousal damage caused by excessive biliary secretion of SN-38 is one of the most important factor culminating diarrhea (Mathijssen, van Alphen et al. 2001). Similarly, low glucuronidation of SN-38 in intestine causes higher amount of SN-38 to be exposed to the intestinal epithelial cells for a long time which is another contributing factor to increase the chance of irinotecan induced late diarrhea (Gagne, Montminy et al. 2002; Han, Lim et al. 2009). In addition, reactivation of toxic SN-38 from SN-38G by bacterial β glucuronidase also increases the chance of late onset diarrhea (Kehrer, Sparreboom et al. 2001). Lastly, direct transformation of irinotecan into SN-38 in gut leads to elevation of SN-38 content which destroys the intestinal epithelial cells leading to delayed severe diarrhea (Hecht 1998; Ahmed, Vyas et al. 1999). Multiple factors such as presence of intestinal CES, activity of bacterial-β-glucuronidase as well as efflux transporters and enterohepatic recycling of irinotecan and SN-38 increase the intestinal concentration of SN-38 leading to SN-38 induced cytotoxicity. Lastly, administration of irinotecan leads to the enhancement of PGE2, TXA2 and COX-2 expression in rat colon which plays an important role to cause electrolyte imbalanace in colon (Sakai, Diener et al. 1995; Kase, Hayakawa et al. 1997; Sakai, Sato et al. 1997). In addition, irinotecan also increases

different inflammatory mediators such as cytokines (TNF-α, IL-1β, IL-6 etc.), nuclear factor-κb and myeloperoxidase activity in intestinal tissues which may be speculated to be involved in the pathogenesis of irinotecan induced late onset diarrhea.

Table 1: Common Toxicity Criteria for diarrhea, Redrawn from National Cancer Institute

Grade	Description
1	Increase of <4 stools per day over baseline; mild increase in ostomy output compared to baseline
2	Increase of 4-6 stools per day over baseline; moderate increase in ostomy output compared to baseline
3	Increase of >7 stools per day over baseline; incontinence; hospitalization indicated; severe increase in ostomy output compared to baseline; limiting self care ADL (Activities of Daily Living)
4	Life threatening consequences; urgent intervention indicated
5	Death

Neutropenia is lack of a type of white blood cells called neutrophils. Absence of neutrophils for a long time leads to the decreased function of immune systems resulting in life threatening condition. Apart from diarrhea, one of the reason for discontinuing the irinotecan treatment is the incidence of neutropenia in a subset of irinotecan treated patient population. Like diarrhea, inefficient metabolism and excretion of SN-38 is considered as one of the primary factor which causes deficiency of neutrophils in irinotecan treated patients. Elevated plasma concentration of SN-38 is regarded as one of the major factor which increases the chance of neutropenia. Pharmacogenetic studies indicated that genetic polymorphism of UGT1A1 enzyme is directly linked with the incidence of neutropenia (Tukey and Strassburg 2000; Ichikawa, Uehara et al. 2015). In general, UGT1A1*28 polymorphism in Caucasian and UGT1A1*6 polymorphism in Asian population causes decreased production of SN-38 glucuronide leading to elevation of plasma concentration of SN-38 which results in neutropenia. To decrease the incidence of neutropenia due to irinotecan treatment, FDA has recommended to physicians to determine a patient's genotype prior to initiate the chemotherapy. In addition, FDA also provided guidelines to determine the optimum dose for a given genotype in order to prevent the toxicity (Ichikawa, Uehara et al. 2015).

1.5. Pharmacokinetic and disposition profile of irinotecan

1.5.1. Plasma pharmacokinetics of irinotecan

Till date the plasma pharmacokinetic profile of irinotecan as well as SN-38 has been well studied (Chabot 1997; Gupta, Mick et al. 1997; Xie, Mathijssen et al. 2002). In human, after intravenous administration of irinotecan (125 to 350mg/m^2 dose range), the peak

plasma conc. (C_{max}) of irinotecan reached to 1 to 10mg/L. Though the area under curve (AUC) of both irinotecan and SN-38 were linear with the increment of dose, marked interpatient variability was observed for both compounds. Pharmacokinetic studies showed the terminal half life ($t_{1/2}$) of irinotecan and SN-38 was 13 h and 17-25 h, respectively (Gupta, Mick et al. 1997; Xie, Mathijssen et al. 2002). Pharmacokinetic profile of irinotecan can be described by a two compartment model which described the range of volume of distribution (Vss) from 136 to 445 L/m^2 and total body clearance (Cl) about 8 – 20 L/h/m^2. Both irinotecan and SN-38 showed moderate to strong binding with the human plasma (Burke and Mi 1994; Combes, Barre et al. 2000). Irinotecan exhibited approximately 60% binding over 0.1 to 4 mg/ml, whereas SN-38 showed comparatively stronger binding about 95% over 0.05 to 0.2 mg/ml. For both compounds, human serum albumin has been found as the major binding plasma protein (Burke and Mi 1994; Schoemaker, Kuppens et al. 2005). In comparison to irinotecan, most of the SN-38 was located in blood cells (almost 66%). Apart from intravenous route, few clinical pharmacokinetic studies have been conducted using oral delivery of irinotecan which showed the AUC of irinotecan and SN-38 increased linearly with dose (Drengler, Kuhn et al. 1999; Schoemaker, Kuppens et al. 2005). But low bioavailability (<20%) and irregularity in AUCs (>50%) precluded the oral delivery of irinotecan.

1.5.2. Metabolism and transport

Metabolic profile of irinotecan is quite complicated one and involves various kinds of drug metabolizing enzymes and efflux transporters. After intravenous administration, irinotecan generates its active metabolite SN-38 which is 100 – 1000 times more potent

than irinotecan in terms of cytotoxic action (Mathijssen, van Alphen et al. 2001). Due to the poor solubility of SN-38, irinotecan is utilized as prodrug in clinical settings. After intravenous administration, the carbamate bond between the dipiperidino side chain and camptothecin moiety of irinotecan is cleaved by carboxylesterase enzymes in liver (Sanghani, Quinney et al. 2004). Among all of the isoforms of CES, CES2 is found to be the most efficient and potent enzyme which mediate the hydrolysis reaction efficiently. Researchers demonstrated that presence of CES at liver and intestine is crucial in irinotecan associated intestinal toxicity as depending on the availability of CES enzymes, SN-38 content in colon increases over time (Ahmed, Vyas et al. 1999; Sanghani, Quinney et al. 2004). In addition, the pattern of bioactivation of SN-38 as well as gut toxicity also differs significantly owing to the significant interindividual variation of CES expression (Sanghani, Quinney et al. 2004).

Apart from CES, another drug metabolizing enzyme which participates actively in the intestinal toxicity of irinotecan is UGT. Several UGT isoforms like UGT1A1, UGT1A9, and UGT2B7 etc. have the ability to produce inactive and non-toxic SN-38 glucuronide from SN-38 (Iyer, King et al. 1998; Ciotti, Basu et al. 1999; Gagne, Montminy et al. 2002). Among all isoforms of UGT, UGT1A1 is the most active isoform which mediates glucuronidation of SN-38. However, SN-38 can also be regenerated from SN-38 glucuronide by bacterial β glucuronidase secreted from commensal microbiota (Catimel, Chabot et al. 1995). But studies indicated that both SN-38 and SN-38 glucuronide can undergo enterohepatic recycling (Catimel, Chabot et al. 1995).

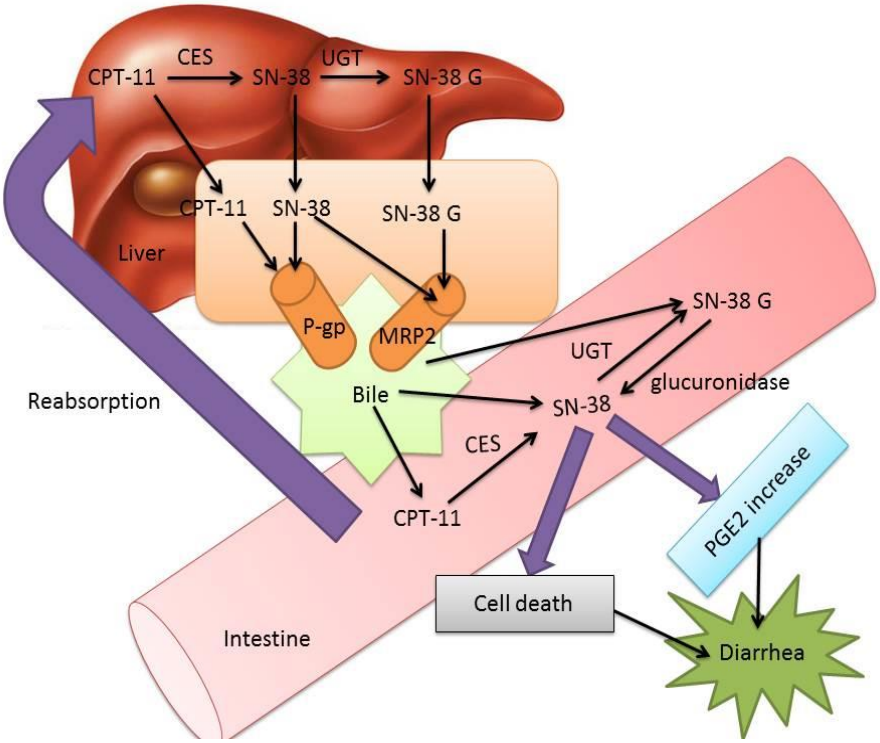


Figure 1: Schematic of key events in the disposition and intestinal toxicity of the irinotecan (CPT-11). After uptake into hepatocytes, carboxyesterase (CES) converts the prodrug CPT-11 to its active metabolite SN-38 which is conjugated by a UGT isozyme to the glucuronide metabolite SN-38G. Secretion of CPT-11, SN-38 and SN-38G is mediated by efflux transporters P-gp and MRP2. Once in the intestinal lumen, SN-38G can be deconjugated by bacterial β-glucuronidase and CPT-11 can be converted to SN-38 by carboxyesterase (CES). SN-38 can be reabsorbed for another cycle of enterohepatic circulation. Alternatively, SN-38 can travel distally and stimulate late-onset diarrhea either by its cytotoxic poisoning of cytotoxic metabolite SN-38 or by stimulating prostaglandin mediators (PGE2) which disturb fluid absorption.

Irinotecan also undergoes oxidation mediated by phase I reaction cytochrome P-450 and forms two oxidative products known as APC (7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin) and NPC (7-ethyl-10-(4-amino-1-piperidino) carbonyloxycamptothecin). Both APC and NPC lack cytotoxicity, whereas NPC can be hydrolyzed to SN-38 by human hepatic and plasma carboxylesterases (Dodds, Haaz et al. 1998; Haaz, Rivory et al. 1998). Among all CYPs, mainly CYP3A4 and CYP3A5 mediate the oxidation of irinotecan (Santos, Zanetta et al. 2000). Recent evidences suggest that almost all part of NPC completely converts to SN-38 in the systemtic circulation (Lokiec, du Sorbier et al. 1996).

Till date several efflux transporters have been found to exhibit transport of irinotecan and SN-38. In tumor cells, both p-glycoprotein (P-gp) and multidrug resistant protein 2 (MRP2)/canalicular multispecific organic anion transporter (cMOAT) demonstrated to mediate the efflux of irinotecan and SN-38 rendering the inrinotecan chemotherapy ineffective (Smith, Figg et al. 2006). Cellular experiments using P-gp and MRP2 overexpressed cells indicated that the cellular accumulation of irinotecan and SN-38 decreased considerably (Sugiyama, Kato et al. 1998). Apart from these two efflux transporters, breast cancer resistant protein (BCRP) transporter was also found to confer resistance to both irinotecan and SN-38 (Yang, Horton et al. 1995). However, till date the mechanisms for the intestinal and biliary transport of irinotecan and its metabolites have not been fully defined.

1.5.3. Excretion

Human excretion studies indicated that irinotecan predominantly eliminates through feces as unchanged form (about 64% of the total dose) (Sparreboom, de Jonge et al. 1998; Slatter, Schaaf et al. 2000). Apart from irinotecan, small amount of SN-38 and the oxidative product APC is also seen to excrete through feces (Gupta, Lestingi et al. 1994; Slatter, Schaaf et al. 2000). The same trend is observed in case of rats where 55% of irinotecan, 10% of SN-38 and 22% of SN-38 glucuronide are excreted over 24 hour (Itoh, Takemoto et al. 2004). The same study also tells us that irinotecan is predominantly eliminated in feces as unchanged form and to some extent in its active metabolite form SN-38.

Both irinotecan and SN-38 can be reabsorbed after undergoing enterohepatic circulation from the intestine following biliary excretion resulting in secondary peak in the plasma concentration-time curve (Gupta, Lestingi et al. 1994; Sparreboom, de Jonge et al. 1998). After start of the i.v. administration, plasma concentrations of SN-38 gradually reached to peak levels within 3 hour and slowly declined after that. However, in some patients after 7-9 hours, a secondary peak is appeared to be seen which increased the AUC of SN-38 by 10-20%. Generally, SN-38 is released from SN-38 glucuronide due to intestinal β glucuronidase mediated hydrolysis and then reabsorbed again. It has been hypothesized that deconjugation and reabsorption may contribute to the variation in pharmacokinetic parameters of irinotecan and SN-38 as well as elevated SN-38 content in colon which leads to severe late onset diarrhea (Gupta, Lestingi et al. 1994; Takasuna, Kasai et al. 1995). Owing to the potential recycling of SN-38, the effective

clearance of SN-38 is minimized which emphasized the clinical significance of high fecal SN-38 concnetration.

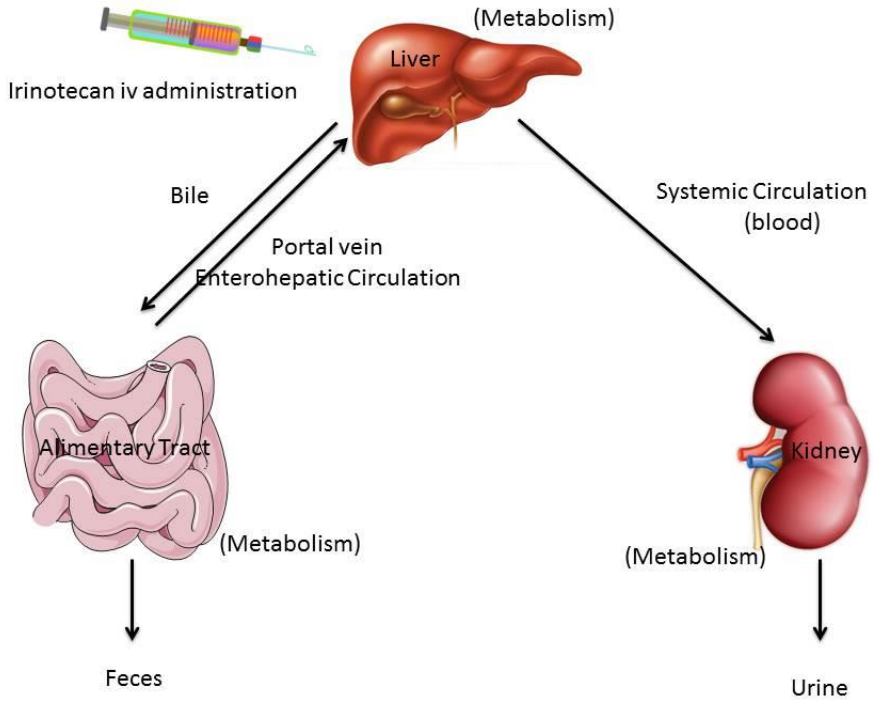


Figure 2: Clearance profile of irinotecan after intravenous administration (Redrawn from Yang et al.)

1.6. Previous attempts to alleviate irinotecan induced toxicity

To prevent irinotecan induced intestinal toxicity different strategies have been hypothesized and tested in preclinical and clinical studies. Among them, the best strategy to prevent irinotecan induced diarrhea is to take preventive measures or prophylactic strategies which consequently improves the safety profile of the drug as

well as quality of the life. Strategies which have been so far devised to alleviate irinotecan induced toxicity are described as follows.

1.6.1. Schedule and dose adjustment

Among all the strategies, this is the first one which was applied to prevent irinotecan induced diarrhea. Preclinical studies showed that at the same dose the incidence of severe diarrhea is quite high in single dose of irinotecan as compared to the protracted schedule (Kurita, Kado et al. 2003). For an example, dose of 60 mg/kg once daily for 4 days induces severe diarrhea, whereas 30 mg/kg for 8 days and 40 mg/kg for 6 days hardly causes any sign of diarrhea. Similarly with the same total dose, extension of infusion time can also reduce the incidence of late onset diarrhea by decreasing the C_{max} of irinotecan (Kurita, Kado et al. 2000). Though, these approaches have lowered the magnitude of toxicities, decreased C_{max} of irinotecan leads to the reduced cytotoxic action which consequently lowers the effectiveness of the chemotherapy. In addition, clinical trial study of low dose continuous intravenous infusion of irinotecan also causes grade 3 adverse event of diarrhea (Herben, Schellens et al. 1999; Takimoto, Morrison et al. 2000).

1.6.2. Structural/chemical modification and drug delivery methods

To decrease the incidence of late onset diarrhea as well as to increase the anticancer activity of irinotecan, different novel approaches has been taken recently. For an example, different formulations of irinotecan and SN-38 such as liposome, nanoparticle, monoclonal antibody conjugate, macromolecular complex etc. have been prepared and tested in different preclinical models (Kojima, Hackett et al. 1998; Zhang, Xuan et al.

2004; Hamaguchi, Doi et al. 2010; Azim and Awada 2012; Kurzrock, Goel et al. 2012). In addition, gene directed enzyme/prodrug therapy and adenovirus mediated β-glucuronidase have also been utilized to increase irinotecan associated chemotherapeutic efficacy as well as to alleviate the severity of diarrhea (Kojima, Hackett et al. 1998). Though there is continuous efforts are going on to develop novel structural analogs and drug delivery systems of irinotecan and SN-38 from last decade, still there is no permanent clinical solution is available to prevent irinotecan associated gut toxicity.

1.6.3. Intetinal alkalization

One of the common strategies to alleviate irinotecan induced intestinal toxicity is utilized in clinical practice is utilization of alkanizing agent in irinotecan treated patients. Generally, all the camptothecin derivatives possess a labile α-hydroxy-δ-lactone ring which undergoes pH-dependent reversible hydrolysis and forms less toxic carboxylate form at physiological pH while at acidic pH lactone form of those derivatives prevails. As the acidic form is more potent and toxic in nature, so alteration of pH at intestinal region might reduce the incidence of diarrhea after shifting the equilibrium from toxic lactone to the non-toxic carboxylate form in the intestine. Different preclinical and clinical studies showed that oral sodium bicarbonate supplement, alkaline ionized water, magnesium oxide etc. facilitates significant benefits in terms of prevention of irinotecan induced late onset diarrhea in different animal (Syrian hamster model, gunn rats etc.) and clinical studies (Takeda, Kobayashi et al. 2001; Tamura, Yasutake et al. 2004; Valenti Moreno, Brunet Vidal et al. 2006). The only disadvantage of this method is the requirement of

daily consumption of highly alkalized water in excess of 2-3 L/day for the entire course of the therapy.

1.6.4. Antidiarrheal agents

Apart from alkalinizing agents, different antidiarrheal agents are also being used to treat irinotecan induced intestinal toxicity in clinical setting. Antimotility drugs such as lopermaide and octreotide are used as the first line and second line therapy for treating diarrhea, respectively (Abigerges, Chabot et al. 1995; Benson, Ajani et al. 2004). Alternatively, acetorphan and budesonide have also shown promising results to ameliorate the severity of late onset diarrhea (Lenfers, Loeffler et al. 1999; Ychou, Douillard et al. 2000).

1.6.5. Alteration of intestinal microbiota

As we described previously, intestinal microflora also plays an important factor to cause intestinal toxicity of irinotecan. Modulation of β-glucuronidase activity in gut may be utilized as one of the promising approach to reduce the severity of diarrhea. In this regard, oral antibiotics were found to be effective to reduce the severity of irinotecan induced gastrointestinal toxicity in both preclinical and clinical studies. Different antibiotics individually or in combination (penicillin, streptomycin, ciprofloxacin) significantly ameliorated the late onset diarrhea and reduced cecal damage without altering pharmacokinetic profile of irinotecan, SN-38 and SN-38 glucuronide in blood, tissues and small intestine (Takasuna, Hagiwara et al. 1996; Takasuna, Hagiwara et al. 1998; Kurita, Kado et al. 2011). Activity of antibiotics is ascribed to a number of factors such as decrease of luminal β glucuronidase activity, inhibition of irinotecan absorption

from intestinal lumen, decrease of CES activity and increase of UGT activity in the intestinal epithelium etc. Extensive usage of antibiotics is limited due to their drastic side effect, negative impact on intestinal metabolism and imbalance of intestinal microflora.

1.6.6. Modulation of metabolizing enzyme and transporter activity

As we already stated that the disposition profile of irinotecan is quite complex and as it involves various types of enymes and transporters, so modulation of the activity of the enzymes and transporters might be another viable strategy to reduce the SN-38 content in colon which consequently will reduce irinotecan induced late onset diarrhea.

1.6.6.1 Enzyme induction/inhibition

In terms of enzyme modulation, so far the most popular strategy is to inhibit the bacterial β-glucuronidase, secreted from commensal microbiota, which increases SN-38 content in colon after mediating the conversion of SN-38 from inactive SN-38 glucuronide in the intestine which ultimately leads to the delayed diarrhea (Ciotti, Basu et al. 1999). Till date, various chemicals have been tried to inhibit the activity of bacterial β-glucuronidase to reduce the conversion of SN-38 from inactive SN-38 glucuronide. Previous studies showed that kampo medications (Japaneese/Chinese herbal) have the ability to reduce enterohepatic circulation of SN-38. Kampo medicines contain four natural glucuronides (baicalin, luteolin, glycyrrhizin and luteolin-3'-glucuronide) which have the ability of inhibiting β-glucuronidase activity. In addition, aglycones of these glucuronide have the ability to inhibit UGT activity towards SN-38 as a substrate. Preclinical studies indicated that administration of kampo medicines such as Hange-Shashin-to (TJ-14) and Dai-kenchu-to can reduce the event of late onset diarrhea in male wistar rats (Narita, Nagai

et al. 1993; Yokoi, Narita et al. 1995). Particularly, TJ-14 can also decrease the colonic prostaglandin E2 (PGE2) production, increase water absorption and protect against irinotecan induced intestinal epithelial damage. In clinical setting, no incidence of grade 3 or 4 diarrhea has been reported after oral administration of TJ-14 to irinotecan treated patients, though constipation due to TJ-14 treatment has been observed in some patients. Like kampo medicines, D-saccharic acid 1, 4-lactone, significantly inhibited irinotecan induced mucosal damage in wistar rats by inhibiting the action of β-glucuronidase activity (Fittkau, Voigt et al. 2004). Recently, a new targeted approach has been taken to inhibit the bacterial β-glucuronidase activity without abolishing the commensal microbiota or disturbing the activity of mammalian β-glucuronidase activity. Wallace et al., demonstrated the presence of a unique specific 17 residue bacterial loop in E. coli enzyme which isn't present in human ortholog (Wallace, Wang et al. 2010). In the same study, he also synthesized inhibitors which are found to exert protective action against irinotecan induced intestinal epithelial damage in male Balb/cJ mice. Both *in vitro* and *in vivo* studies revealed that those inhibitors are effective against bacterial β-glucuronidase activity only without disturbing mammalian β-glucuronidase enzymes. Clinical studies are being pursued to test the efficacy of those synthesized inhibitors.

SN-38 is primarily glucuronidated by UGT1A1 in the liver to form inactive, nontoxic SN-38 glucuronide (SN-38G). So, induction of UGT1A1 might lead to decrease the occurrence of late onset diarrhea after reducing SN-38 content in colon without affecting the systemic content of SN-38. Different agents which can induce UGT1A1 gene have been used in preclinical and clinical studies. Among them, only chrysin and

phenobarbital were able to decrease the event of late onset diarrhea to a significant extent without altering the efficacy profile (Innocenti, Undevia et al. 2004; Tobin, Beale et al. 2006).

Apart from β-glucuronidase and UGT, CES also contributes significantly in the disposition of irinotecan. It is speculated that activation of biliary excreted irinotecan to SN-38 in the small intestine by human intestinal carboxylesterase is one of the major contributing factor which damages intestinal epithelium causing late onset of diarrhea. So inhibition of carboxylesterase enzyme might be a useful strategy to reduce SN-38 content in small intestine. Hicks et al., synthesized some human CES selective sulfonamide derivatives (benzyl, dimethylbenzil etc.) which can also inhibit CES intracellularly (Hicks, Hyatt et al. 2009). In addition, a series of novel, highly potent human CES inhibitors have been designed (Yoon, Hyatt et al. 2004). However, both of these classes of compounds are yet to use in preclinical and clinical studies.

There are reports where modulation of different enzymes other than β-glucuronidase, UGT and CES have been performed to reduce the irinotecan induced intestinal toxicity. Though different CYP3A4 inducers (phenytoin, carbamazepine, phenobarbital etc.) were found to increase the irinotecan and SN-38 clearance, none of them actually reduced the event of diarrhea (Prados, Yung et al. 2004). On the other hand, COX-2 inhibitor celecoxib was found to decrease the severity of irinotecan induced diarrhea in different animal models (Sprague dawley and ward CRC rat model) (Trifan, Durham et al. 2002; Javle, Cao et al. 2007). As it is hypothesized that the administration of irinotecan is associated with the increased PGE2 and TXA2 which contributes to the secretory

diarrhea, so COX-2 inhibitors might be utilized as an alternative option to reduce late onset diarrhea in clinical settings. Multiple clinical studies have been conducted using celecoxib, but none of them prevented inirinotecan induced diarrhea. The explanation of the failure of celecoxib treatment might be either inhibiting COX-2 alone is not sufficient enough to ameliorate the diarrhea or the amount of celecoxib reaching at the target tissues isn't sufficient enough to exert the COX-2 inhibitory effect.

1.6.6.2 Efflux transporter inhibition

Transport of irinotecan and its metabolites have been regulated by different efflux transporters like P-gp, MRP2 and BCRP. As a significant portion of SN-38 and its glucuronide is transported by various efflux transporters, so inhibition of these efflux transporters is a viable option to reduce biliary concentration of SN-38 which will ultimately lead to decrease of late onset diarrhea. Irinotecan, SN-38 and its phase II metabolite SN-38 glucuronide is effluxed to the biliary tract from systemic circulation by P-gp and MRP2 transporters and enter into the eneterohepatic recycling which prolongs their duration in the body as well as increase the chance of intestinal toxicity. Preclinical studies have shown that cyclosporine A, an inhibitor of both P-gp and MRP2 transporters, decreased the biliary excretion of the SN-38 and SN-38 glucuronide in preclinical studies (Gupta, Safa et al. 1996). Subsequent clinical studies also revealed that coadministration of cyclosporine A can decrease the incidence of grade 2/3/4 diarrhea (Chester, Joel et al. 2003; Vasudev, Jagdev et al. 2005). However, as cyclosporine A has severe side effects (nephrotoxicity, hepatotoxicity, neuropathy etc.) so its usage has been restricted in clinical settings. Apart from cyclosporine A, other

substances like verapamil and quercetin was also found to decrease biliary excretion of irinotecan as well as SN-38 in female wistar rats (Bansal, Awasthi et al. 2008; Bansal, Mishra et al. 2009). Studies indicated that MRP2 have more impact on the efflux of SN-38 and SN-38 glucuronide in comparison to irinotecan. Coadministration of MRP2 specific inhibitor probenecid also decreased the biliary excretion of irinotecan, SN-38 and SN-38 glucuronide in rats as well as reduced irinotecan induced intestinal toxicity (Mathijssen, van Alphen et al. 2001; Horikawa, Kato et al. 2002). Like cyclosporine A, the usage of probenecid in clinical setting is limited due to its side effect, e.g. nephrotoxicity. In comparison to P-gp and MRP2, BCRP has little contribution in the disposition of irinotecan; although one BCRP specific inhibitor GF120918 was found to decrease irinotecan induced intestinal damage (Zhang, Pan et al. 2008).

1.7. PHY 906 and Xiao Chao Hu Tang

PHY906 is a novel Chinese herbal preparation which demonstrates a wide range of pharmacological activities such as antiviral, immunologic, analgesic, vasodilatory, hepatoprotective, antioxidant, and appetite stimulatory effects (Kummar, Copur et al. 2011). This particular preparation is composed of 4 main herbs, *Scutelleria baicalensis Georgi, Paeonia lactiflora Pall.,Glycyrrhiza uralensis Fisch.,* and *Ziziphus jujuba Mill*. PHY 906 is extensively used to treat a variety of gastrointestinal side effects such as diarrhea, abdominal cramps, vomiting, as well as fever and headache (Kummar, Copur et al. 2011). Owing to its cryoprotective effect and lack of severe side effects, it has also been used in preclinical and clinical studies to investigate whether it can reverse irinotecan induced late onset diarrhea. It is found to decrease the incidence of late onset

diarrhea as well as to potentiate the anticancer activity of irinotecan by promoting the expression of intestinal stem cells (Lam, Bussom et al. 2010; Kummar, Copur et al. 2011). A phase I trial of PHY 906 with bolus weekly regimen of irinotecan, 5-flurorouracil, and leucovrin (IFL) has been conducted on advanced colorectal cancer patients which reported that PHY906 doesn't alter the pharmacokinetic profile of irinotecan. The same study also indicated that PHY 906 can improve the condition of patients by preventing the grade 3/4 diarhhea (Lam, Bussom et al. 2010; Kummar, Copur et al. 2011). However, larger randomized studies are needed to evaluate the benefit of PHY 906 in irinotecan induced late onset diarrhea as previous trial had relatively small number of patients. On the other hand, quality control of different components in the herb is one of the major concern in clinical settings which is to some extent was resolved by frequently employing high performance liquid chromatography (HPLC) and liquid/gas chromatography-mass spectroscopy (LCMS). However, in order to receive the marketing approval from regulatory authorities an appropriate phase II and III study is required.

Like PHY 906, Chinese medicine Xiaochaihutang (XCHT, known as Sho-Saiko-To or SST or TJ-9 in Japan, Minor bupleurum decoction) is also undergoing clinical trial to get the approval from regulatory authorities for the treatment of chronic hepatitis (Song, Kim et al. 2014; Takahashi, Soejima et al. 2014). This formulation is mainly composed of seven herbs namely, *Radix Bupleuri*, *Radix Scutellariae*, *Rhizoma Pinelliae*, *Radix Ginseng*, *Radix Glycyrrhizae*, *Rhizoma Zingiberis Recens*, and *Fructus Jujubae*. Different active components such as liquiritin, baicalein, baicalin, wogonin, wogonoside, zingerone, 6-gingerol etc. have been identified in XCHT. This herbal formulation is

mainly used to treat fever, malaria, gastrointestinal disorders and chronic liver diseases. In Japan, a prescription form of XCHT has been used extensively to treat hepatitis. Preclinical studies indicated that XCHT and its main chemical components such as baicalin, baicalein, glycyrrhizin, and saikosaponin-D demonstrated antiproliferative activity against hepatocellular carcinoma, decreased hepatic fibrosis and regenerated liver expression in animal models (Dai, Yang et al. 2008; Song, Kim et al. 2014; Takahashi, Soejima et al. 2014). Human studies also showed reduced incidence of hepatocellular carcinoma in users of ginseng which is one of the herbal component of this formulation (Oka, Yamamoto et al. 1995). *In vitro* studies reported that both XCHT and its isolated chemical components possess marked antiproliferative effects. Preclinical studies confirmed that XCHT can enhance various aspects of immune function, including effects on killer cells, interleukins, interferon and macrophages (Kakumu, Yoshioka et al. 1991; Kaneko, Kawakita et al. 1994; Yamashiki, Nishimura et al. 1997; Nose, Tamura et al. 2003). A clinical study shows XCHT may improve liver pathology in hepatitis C patients who do not respond to interferon-based treatment (Deng, Kurtz et al. 2011). XCHT treated rats generated less fibrosis as indicated by reduced liver hydroxyproline and a smaller increase in serum hyaluronic acid. Moreover, these rats develop fewer preneoplastic lesions (Sakaida, Matsumura et al. 1998). A recent study showed that it also regulates temporal gene expression in XCHT treated mouse hepatocytes by way of microRNA (Song, Kim et al. 2014). Regarding interaction with phase I drug metabolizing enzymes, XCHT upregulated the enzyme expression of CYP2B, CYP3A1 and CYP4A1 and subsequently can alter the plasma concentration of these drugs (Nose, Tamura et al. 2003). It also interacts with

drugs metabolized by CYP3A4, CYP2C9 and CYP1A2 enzymes (Takahashi, Uejima et al. 2003). Owing to the safe profile of XCHT, various preclinical and clinical studies are going on involving XCHT at present.

1.8. Summary

Irinotecan is widely used anticancer drug which demonstrated grade 3/4 late onset diarrhea in approximately 30-40% of patient population. Till date, there is no permanent clinical solution available to prevent irinotecan induced late diarrhea. It has been hypothesized that elevation of colonic SN-38 which is an active metabolite of irinotecan is responsible for causing late onset diarrhea. In this project, we systematically investigated different mechanisms which might reduce the colonic exposure of SN-38 resulting in amelioration of diarrhea.

Table 2: Summary of approaches have been taken to alleviate irinotecan induced late onset diarrhea

Approaches	Purpose	Observation & Comments
Schedule and dose adjustment	To decrease the total SN-38 content in intestinal lumen by protracting the dosing schedule and extending the infusion time.	Successful in most of the cases, but chance of loss of efficacy of chemotherapy. Grade 3 diarrhea is seen in some of the cases. (Kurita et al., 2000, Kurita et al., 2003, Takimoto et al., 2000)
Structural/chemical changes and Novel drug deliver systems	Different structural modification through chemical reaction have been tried to reduce the SN-38 exposure as well as to improve the chemotherapeutic efficacy. In addition, different novel formulation such as liposome, nanoparticle and monoclonal antibody have been tried.	Though some preclinical *in vitro* results are encouraging, no *in vivo* preclinical results are available for chemically modified irinotecan/SN-38 analogues. For some of the chemical congeners efficacy isn't established. (Kozima et al., 1998, Zhang et al., 2004, Hamaguchi et al., 2010, Azim et al., 2012)
Intestinal alkalization	To decrease the lactone form of SN-38 in the body which is relatively more toxic than the carboxylate form.	Sodium bicarbonate supplement, alkaline ionized water, magnesium oxide etc. have the ability to prevent irinotecan induced late onset diarrhea in different animal models and clinical studies. But daily consumption of highly alkalized water in excess of 2-3 L/day is required during the therapy. (Takeda et al., 2001, Tamura et al., 2004, Valenti et al., 2006)
Antidiarrheal agents	Antimotility drug Loperamide, somatostatic agent octreotide, antidiarrheal drug acetorphan, anti-inflammatory agent budenoside etc. have been used to prolong the intestinal transit time.	Loperamide and octeotride treatment is currently utilized as first and second line of treatment. But decreased intestinal transit may cause more mucosal damage. Though acetorphan is not effective as prophylactic treatment, budenoside exhibits some promising result. However its clinical side effect is still unknown. (Abigerges et al., 1995, Benson et al., 2005, Lenfers et al., 1999, Ychou et al., 2000)

Alteration of intestinal microbiota	Different antibiotics such as ciprofloxacin, penicillin, neomycin etc. were utilized to prevent/reverse irinotecan induced late onset diarrhea symptomatically.	Antibiotics effectively decrease the incidence of irinotecan induced late onset diarrhea through decreasing luminal β glucuronidase activity and inhibiting carboxylesterase activity. But extensive usage of antibiotic is limited as they have drastic side effects and negative effect on intestinal metabolism. (Takasuna et al., 1996, Takasuna et al., 1998, Kurita et al., 2011)
Inhibition of efflux transporter	Various chemicals/drugs such as cyclosporine A, probenecid, quercetin, phenobarbital etc. were used to inhibit biliary/intestinal excretion of irinotecan and SN-38 mediated by P-gp, MRP2 etc.	Though most of the drugs/chemicals show promising result in preclinical studies, none of them can be utilized clinically owing to their adverse effects or lack of translating the activity in clinical setting. (Gupta et al.,1996,Bansa et al., 2009, Horikawa et al., 2002)
Inhibition of β glucuronidase enzyme activity	Different herbal products and chemicals such as TJ14, kampo medicines have been tried to inhibit β glucuronidase activity to reduce the regeneration of SN-38 from SN-38 glucuronide	Exhibited encouraging preclinical results but clinically not proven yet. Quality control of the herbal products is a major concern. (Narita et al., 1993, Narita et al., 1995, Fittkau et al., 2004, Wallace et al., 2010)
Inhibition of carboxylesterase enzyme activity	Synthetic compounds (Benzil) were used to inhibit intestinal carboxylesterase activity to reduce the formation of SN-38 from irinotecan	*In vitro* cellular study showed these compounds are active, though they aren't tested yet in animal model. (Yoon et al., 2004, Hicks et al., 2009)
Induction of UGT enzymes	Chrysin and phenobarbital was used to induce UGT1A1 activity to elevate inactive SN-38 glucuronide formation from toxic SN-38	Approximately 10% of patient population didn't respond to chrysin. Phase II clinical trial is going. (Innocenti et al., 2004, Tobin et al., 2006)
COX-2 and PGE2 inhibition	Celecoxib was used to treat the late onset diarrhea by inhibiting COX-2 and PGE2 activity	Encouraging preclinical study results didn't translate to clinical settings. (Trifan et al., 2002, Javde et al., 2007)
Decreased irinotecan absorption	Activated charcoal and AST-120 have been used to decrease the irinotecan content in intestinal lumen	Showed incomplete absorption, patient non-compliant due to high frequency of drugs. (Chyka 1995; Hidaka, Yamasaki et al. 2007; Sergio, Felix et al. 2008)
Reversing intestinal epithelium damage	Marketed herbal formulation PHY 906 was used to treat late onset diarrhea by promoting the expression of intestinal progenitor/stem cells and increase proliferative stem cells in the intestine	Potentiated antitumor activity and proliferated cells in the intestine after irinotecan treatment despite quality control is a major concern. It warrants larger phase II study (Kummar et al.,2011, Lam et al.,2010)

Chapter 2 Statement of Problems

2.1. Purpose

To decrease the biliary and intestinal excretion of SN-38 and SN-38 glucuronide for reducing colonic SN-38 exposure in order to alleviate irinotecan induced intestinal toxicity.

2.2. Specific aims

Towards our goal, three major specific aims were proposed:

2.2.1. Aim I

To develop a modified *in situ* rat perfusion model for determination of the disposition of irinotecan, SN-38 and SN-38 glucuronide in major metabolic organs and bio-matrices.

In this particular aim, we will develp a modified *in situ* rat perfusion model by which we can determine the disposition of irinotecan, SN-38 and SN-38 glucuronide in major metabolic organs (liver and kidney) and bio-matrices (bile, perfusate, urine and plasma). Development of the modified *in situ* rat perfusion study will allow us to determine the absorption, distribution and excretion of irinotecan and its metabolites which will give us a holistic idea about the disposition profile of the administered irinotecan.

2.2.2. Aim II

To investigate the potential of approved drugs, natural products and herbal formulations to reduce colonic exposure of SN-38 by inhibiting biliary and intestinal excretion of SN-38 and SN-38 glucuronide employing the modified *in situ* rat perfusion model.

In this aim, we will focus on the inhibition potential of approved drugs, natural products and herbal formulations which can actually decrease the colonic content of SN-38 by reducing biliary and intestinal excretion of SN-38 and SN-38 glucuronide. Ultimately this model will be utilized as a screening tool to explore the impact of efflux transporters on the disposition of irinotecan and its metabolites in major metabolic organs and bio-matrices.

2.2.3. Aim III

To determine if the selected agents can decrease the colonic exposure of SN-38 by decreasing fecal content of SN-38 employing *in vivo* pharmacokinetic study.

In this particular aim, we will conduct *in vivo* pharmacokinetic study of irinotecan in presence of selected chemical inhibitor and herbal formulation to observe if they can reduce the fecal excretion of SN-38 without altering the efficacy of the chemotherpautic treatment.

2.2.4. Expected Outcome

In this project, we will systematically investigate the mechanisms which are responsible to reduce the colonic content of SN-38 resulting in amelioriation of late onset diarrhea. To achieve this goal, first we will develop and validate a UPLC-MS/MS method for the simulataneous determination of irinotecan, SN-38 and SN-38 glucuronide in different bio-matrices (plasma, bile, urine and intestinal perfusate) and in major metabolic organs (liver and kidney). Then, we will develop a modified *in situ* rat perfusion model through

which we expect to get a holistic idea about the disposition of irinotecan and its metabolites. Using the same perfusion model, we will screen approved drugs, some herbal formulations and natural agents which will decrease the biliary and intestinal excretion of SN-38 and SN-38 glucuronide. Also, we expect to observe the impact of different efflux transporters on the disposition of irinotecan by conducting the same study. Finally, we will perform pharmacokinetic study to evaluate the effectiveness of the selected approved drug and herbal formulation to decrease the colonic exposure of SN-38 where we expect to see these particular agents can decrease the fecal excretion of SN-38 without altering its plasma profile.

2.2.5. General strategy

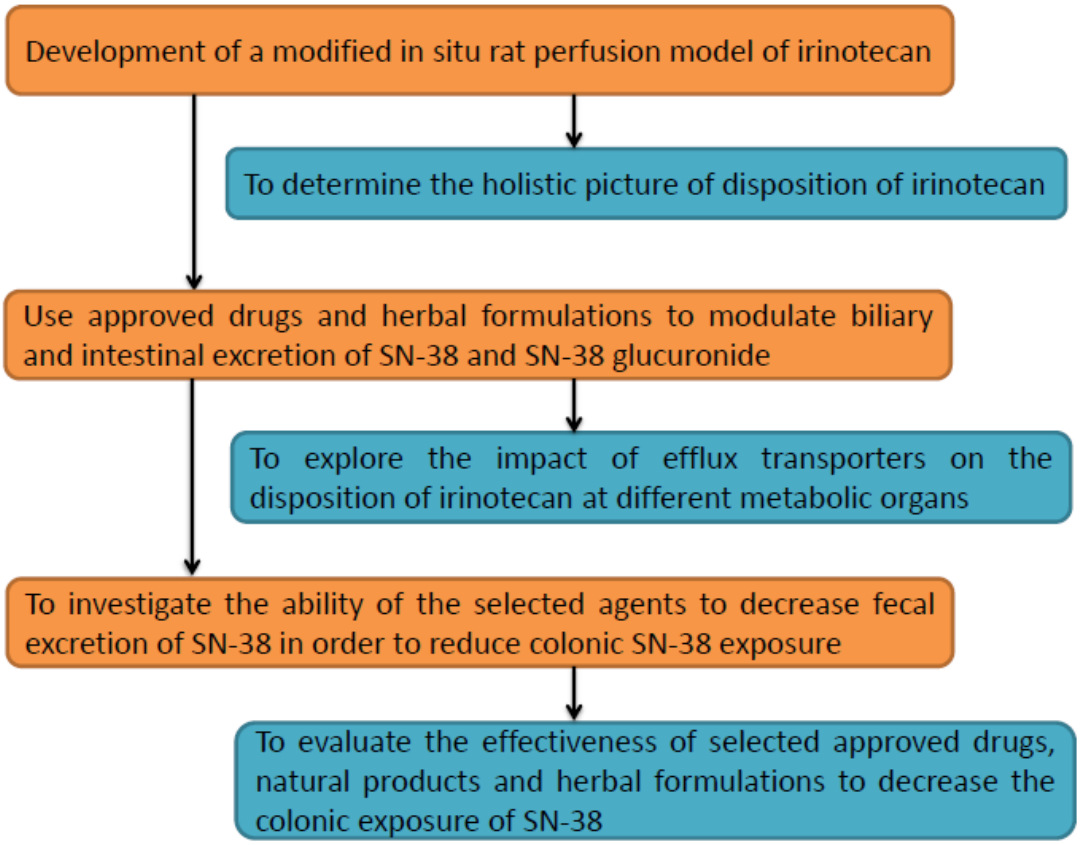


Figure 3: Schemetic diagram of general research strategy

Chapter 3 Development and validation of an UPLC-MS/MS method for the quantification of irinotecan, SN-38 and SN-38 glucuronide in plasma, urine, feces, liver and kidney: Application to a pharmacokinetic study of irinotecan in rats

3.1. Abstract

The objective of this research is to develop and validate a sensitive and reproducible UPLC-MS/MS method to quantify irinotecan, its active metabolite SN-38 and SN-38 glucuronide (phase II metabolite of SN-38) simultaneously in different bio-matrices (plasma, urine, feces), tissues (liver and kidney) and to use the method to investigate its pharmacokinetic behavior in rats. Irinotecan, SN-38 and SN-38 glucuronide has been resolved and separated by C18 column using acetonitrile and 0.1% formic acid in water used as the mobile phases. Triple quadruple mass spectrometer using multiple reaction monitoring (MRM) with positive scan mode were employed to perform mass analysis. The results showed that the linear response range of irinotecan and SN-38 in plasma, feces, liver and kidney is 4.88 -10000 nM, 39 - 5000 nM, 48.8 -6250 nM and 48.8 - 6250 nM, respectively (R^2 > 0.99). In case of SN-38 glucuronide, the standard curves were linear in the concentration range of 6.25 - 2000 nM, 4.88 - 1250 nM, 9.8 – 1250 nM and 9.8 – 1250 nM in plasma, feces, liver and kidney homogenates, respectively. The lower limit of detection (LLOD) of irinotecan, SN-38 and SN-38 glucuronide was determined to be less than 25 nM in all bio-matrices as well as tissue homogenates. Recoveries of irinotecan, SN-38 and SN-38 glucuronide at three different concentrations (low, medium and high) were not less than 85% at three different concentrations in plasma and feces.

The percentage matrix factors in different bio-matrices and tissues were within 20%. The UPLC-MS/MS method was validated with intra-day and inter-day precision of less than 15% in plasma, feces, liver and kidney. Owing to the high sensitivity of this method, only 20 µl plasma, urine and homogenates of liver, kidney and feces is needed. The validated method has been successfully employed for pharmacokinetic evaluation of irinotecan in male wistar rats to quantify irinotecan, SN-38 and SN-38 glucuronide in plasma, feces, and urine samples.

3.2. Introduction

Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecine, CPT-11, Fig. 1 B) is an important component of FOLFIRI and FOLFIRINOX chemotherapy regimen which are used as first line treatment against colorectal and advanced pancreatic cancer (Tournigand, Andre et al. 2004; Akhtar, Chandel et al. 2014). It has also shown clinical activity against other types of cancers such as lung, ovarian, cervical, gastric, refractory lymphoma and leukemia etc. (Ohno, Okada et al. 1990; Fukuoka, Niitani et al. 1992; Shimada, Yoshino et al. 1993) . Irinotecan is a pentacyclic semisynthetic derivative of camptothecin which is isolated from the bark of a tree named *Camptotheca acuminate* in 1966 (Wall M. E. 1966; Sawada, Okajima et al. 1991). Irinotecan, through the action of liver carboxylesterase gets converted into its active metabolite SN-38 (7-ethyl-10-hydroxycamptothecin) which is 100-1000 times potent than irinotecan (Rothenberg, Kuhn et al. 1993; Mathijssen, van Alphen et al. 2001). Anticancer activity of irinotecan is attributed to the inhibitory effect of SN-38 on DNA topoisomerase I which plays an important role in DNA replication and transcription

(Redinbo, Stewart et al. 1998; Stewart, Redinbo et al. 1998). Formation of an irreversible complex of SN-38, DNA topoisomerase I enzyme and the ligated DNA strand leads to the breakage of double stranded DNA which ultimately causes cell death. Owing to the poor solubility of SN-38, irinotecan is currently used as a prodrug in clinical applications. Inactivation of SN-38 to its phase II metabolite SN-38 glucuronide occurs through an enzymatic reaction mediated by the UDP-glucuronosyl transferase 1A1 isoform (UGT1A1).

It has been shown that the intact lactone ring of CPT derivative is an important factor to retain its anticancer activity (Slichenmyer, Rowinsky et al. 1993; Redinbo, Stewart et al. 1998; Stewart, Redinbo et al. 1998). However, the lactone ring of CPT derivatives undergoes a pH sensitive, reversible hydrolysis and converts into carboxylate form at physiologic pH (Sano, Yoshikawa et al. 2003). Boyd et al. showed that pH played an important role in the inter conversion of lactone and carboxylate forms of CPT in the solution. Experiments showed that at pH 3 to 5, lactone is the most stable form of CPT, whereas at pH 9, it rapidly converts into carboxylate form (Boyd, Smyth et al. 2001). Till date, HPLC methods coupled with fluorescence detectors have been the most predominant method for detection of CPT derivatives as they can detect both lactone and carboxylate forms in different biological matrices.

Different bioanalytical techniques, mostly based on reverse phase liquid chromatography coupled with fluorescence detection, have been proposed to determine irinotecan and SN-38 in various biological matrices. However, these fluorescence methods suffered from several drawbacks such as lengthy preparation time, limited sensitivity and high

sample volumes. Recently, liquid chromatography coupled with mass spectrometry (LC-MS and LC-MS/MS) has become one of the preferred analytical tools for the rapid and efficient quantification of small and large molecules in different biological matrices due to the unique combination of high specificity, sensitivity and high sample throughput possibilities. Although several LC-MS/MS methods have been developed and implemented for the quantification of irinotecan and SN-38 in rabbit, mouse and human plasma, very few of them quantified SN-38 glucuronide in plasma (Corona, Elia et al. 2010; Ahn, Park et al. 2014; Park, Won et al. 2014). Similarly, though quantification of irinotecan and SN-38 was done in mouse tissues, the quantification of SN-38 glucuronide in these tissues has not been reported (Bardin, Guo et al. 2005). Also, to the best of our knowledge there is no LC-MS/MS method available to quantify irinotecan, SN-38 and SN-38 glucuronide in urine and feces. In this study, we developed and validated a simple, rapid and sensitive LC-MS/MS method for simultaneous quantification of total concentration of irinotecan, SN-38 and SN-38 glucuronide in rat plasma, feces, urine, liver and kidney homogenate to support pharmacokinetic studies in rats.

3.3. Materials and methods

3.3.1. Materials

Irinotecan, SN-38, CPT, uridine-5'-diphosphate-β,D-glucuronic acid ester (UDPGA), D-saccharic-1,4-lactone monohydrate, magnesium chloride, hanks' balanced salt solution (powder form) and formic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Expressed human UGT isoforms (UGT1A1) was purchased from BD Biosciences

(Woburn, MA, USA). Solid phase extraction (C18) columns were purchased from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile, methanol and water (LC-MS grade) were purchased from EMD (Gibbstown, NJ, USA). Water was deionized by a Milli-Q water purification system of Millipore (Bedford, MA, USA). Intravenous irinotecan hydrochloride injection (20 mg/ml) was purchased from Teva Pharmaceuticals (Pearl River, NY, USA).

3.3.2. Bio synthesis of SN-38 glucuronide

SN-38 glucuronide was biosynthesized using human expressed UGTs (UGT1A1 isoform). In this study, SN-38 was incubated with UGT1A1 for 24 hours which resulted in the formation of 95.8% SN-38 glucuronide. We separated the residual aglycone (SN-38) from the mixture after employing liquid-liquid extraction utilizing dichloromethane (DCM). Then each 5 ml of processed samples were applied to a C18 solid phase extraction column. After washing out the salt, 1 ml of methanol was used to elute the SN-38 glucuronide. The eluted fractions of methanol were collected and air dried, and the residue was reconstituted with 100 µl of 50% methanol-water to concentrate SN-38 glucuronide. In each step, we measured the respective peak area of SN-38 and SN-38 glucuronide in UPLC to calculate the purity of SN-38 glucuronide in the mixture. Apart from UPLC quantification, we also utilized MS/MS spectrum of SN-38 glucuronide to confirm its presence in the solution.

3.3.3. UPLC-MS/MS Study

The chromatographic separation of irinotecan, SN-38 and SN-38 glucuronide was achieved by a Waters Acquity^{TM} UPLC H-Class system with a diode array detector (DAD) and a flow-through-needle sample manager. Analysis was carried out using

Acquity UPLC BEH C18 Column (2.1 mm × 50 mm, 300˚A, 1.7 µm, Waters, Milford, MA, USA). Mobile phase A (0.1% formic acid in water [v/v]) and mobile phase B (100% acetonitrile) were operated with a gradient elution at a flow rate of 0.4 ml/min as follows: 10% B → 25% B (0 – 0.5 min), 25% B → 40% B (0.5 - 1 min), 40% B (1 – 2.5 min), 40% B → 10% B (2.5 – 4.5 min). The column temperature and sample temperature was 60°C and 20°C. The injection volume was 10 µl. 100 nM camptothecin (CPT) in 50% methanol-water was used as internal standard (IS).

LC-MS/MS analysis for irinotecan, SN-38 and SN-38 glucuronide was performed with an API 5500 Qtrap triple quadruple mass spectrometer coupled with a TurboIonSpray™ (Applied Biosystem- MDS SCIEX, Framingham, MA, USA). The system was operated in positive electrospray ionization (ESI) and multiple reactions monitoring (MRM) scan mode. All data were acquired and processed using Analyst®1.5.2 software with hotfixes (AB SCIEX).

3.3.4. Method validation

3.3.4.1 Calibration curve and LLOD

Calibration standards were prepared in 50% aqueous methanolic solution by diluting a stock solution of irinotecan, SN-38 and SN-38 glucuronide to final concentrations of 50, 50 and 10 µM respectively and were kept at −80°C. Working solutions were prepared by serial dilutions of stock solutions with 50% methanol-water at the following concentrations: 6.25, 12.5, 25, 50, 100, 250, 500, 1000 nM of irinotecan; 6.25, 12.5, 25, 50, 100, 250, 500, 1000 nM of SN-38 and 3.12, 6.25, 12.5, 25, 50, 100, 250, 500 nM of SN-38 glucuronide. The standard and quality control solutions (QCs) were prepared by

mixing 20 µl of blank plasma, liver, kidney and fecal homogenates with each 20 µl working solutions of CPT-11, SN-38 and SN-38 glucuronide in 1.5 ml mini-eppendorf tubes. QCs were prepared at the following concentrations: 40, 200 and 1000 nM for CPT-11, 40, 200 and 1000 nM for SN-38 and 8, 40 and 200 nM for SN-38 glucuronide. All sample solutions of rat plasma, feces, liver and kidney homogenates were also added with 20 µl working solutions of CPT (100 nM). Then the solution is extracted with 360 µl methanol-acetonitrile (1:1, [v/v]) solution, vortex-mixed for 1 min and centrifuged at 15,500 rpm for 15 min. The supernatants were transferred to another tube and evaporated to dryness under a steady stream of air at room temperature. The residue was reconstituted with 80 µl of 50% aqueous methanolic solution and centrifuged again at 15,500 rpm for 15 min. After centrifugation, 10 µl of the supernatant was injected to UPLC-MS/MS system for analysis. In case of feces, we followed the same protocol except we added 360 µl acetonitrile to extract irinotecan, SN-38 and SN-38 glucuronide. The linearity of each calibration curve was determined by plotting the ratio of the peak areas of analyte (Irinotecan/SN-38/SN-38 glucuronide) to internal standard (I.S.; CPT in 50% methanol) in rat plasma, feces and homogenized liver and kidney. A least-square linear regression method ($1/x^2$ weight) was used to determine the slope, intercept and correlation coefficient of linear regression method. The lower limit of quantification (LLOQ) was determined based on the signal-to-noise ratio of at least 10:1.

3.3.4.2 Accuracy and precision

The “intra-day” and “inter-day” precision and accuracy of the method were determined with quality control (QC) samples at three different concentrations (six injections for each

concentration) on the same day or on three different days (Matuszewski, Constanzer et al. 2003).

3.3.4.3 Extraction recovery and matrix effect

Extraction recovery of irinotecan, SN-38 and SN-38 glucuronide in different bio-matrices (plasma, feces, liver and kidney homogenate) was calculated by plotting the ratio of the peak areas of analyte (irinotecan/SN-38/SN-38 glucuronide) to internal standard (CPT) in blank rat plasma/feces/liver or kidney homogenate spiked before extraction procedure divided by the ratio of the peak areas of analyte to internal standard for the same quantity of the respective compound spiked into extracted blank matrix. Similarly, matrix effects were calculated by dividing the ratio of the peak area of the compound and internal standard spiked into extracted blank matrix by the ratio of the peak area of same compound and internal standard in neat solution at the same concentration. All these experiments and evaluations were performed according to the recommended validation procedures reported by Matuszewski (Matuszewski, Constanzer et al. 2003).

3.3.4.4 Stability

Short-term (25°C for 4 h), post-processing (20°C for 8 h), long-term (−80°C for 1 month) and three freeze-thaw cycle stabilities of irinotecan, SN-38 and SN-38 glucuronide were determined by analyzing three replicates of QC samples at three different concentrations.

3.3.5. *In vivo* rat pharmacokinetic study

3.3.5.1 Animals

Male Wistar rats (6-10 weeks, Body weight between 250 to 280g, n = 6) were purchased from Harlan Laboratory (Indianapolis, IN). Rats were kept in an environmentally controlled room (temperature: 25 ± 2°C, humidity: 50 ± 5%, 12 h dark-light cycle) for at least 1 week before the experiments. The rats were fasted overnight before the day of the experiment.

3.3.5.2 Animal Experiment design

Irinotecan was administered at a dose of 5 mg/kg via intravenous injection through the tail vein. Blood samples (about 20 -50 µL) were collected in heparinized tubes at 0, 15, 30, 60, 120, 240, 360, 480, and 1440 min post-injection, via tail snip with isoflurane as anesthetic. Urine samples were collected at 4, 8 and 24 hours; whereas feces were collected at 24 hour. Plasma samples were prepared and stored at - 80°C until analysis. The procedures were approved by the University of Houston's Institutional Animal Care and Uses Committee (IACUC).

3.3.5.3 Tissue homogenization

Liver and kidney were excised from the sacrificed rats and stored frozen at −80°C in polypropylene tubes until homogenization. The frozen tissues were thawed, chopped and weighed at 4°C. Accurately 50 mg of chopped tissue was homogenized in 2 ml of ice-cold homogenizing solution (pH 7.4) containing 10 mM potassium phosphate, 250 mM sucrose and 1 mM EDTA dehydrate with a polytron tissue homogenizer. Homogenization was paused 20 s after every 30 s of homogenization at a medium

speed. The homogenization was repeated 3 – 4 times until a uniform homogenate was obtained. Final tissue extract was stored at approximately −80°C prior to analysis. The homogenizer probe was washed sequentially with water, methanol and water after every homogenization.

3.3.5.4 Sample Preparation

Plasma, liver and kidney homogenate samples (20 µl) were spiked with 20 µl of I.S. (CPT in 50% methanol, 100 nM) and vortexed for 1 min after extracted with 50% methanol-acetonitrile solution in the ratio of 1:9:9 (solution: methanol: acetonitrile; v/v). All solutions were vortexed and centrifuged at 15,500 rpm for 15 min. The supernatants were transferred to another tube and evaporated to dryness under a steady stream of air at room temperature. The residue was reconstituted with 80 µl of 50% aqueous methanolic solution and centrifuged again at 15,500 rpm for 15 min. After centrifugation, 10 µl of the supernatant was injected to UPLC-MS/MS system for analysis. In case of feces, at first we lyophilized the feces to evaporate the moisture from the sample. Then we weighed 1 g feces accurately and homogenized it with 10 ml of homogenizing solution. After that we centrifuged it for 5 min to remove heavy particles and take out the supernatant. Then, we spiked 20 µl fecal homogenate solution with 20 µl of I.S. (CPT in 50% methanol, 100 nM) and vortexed for 1 min after extracted with acetonitrile solution in the ratio of 1:18 (solution: acetonitrile; v/v). In case of urine, we diluted all the standard and samples 100 times with 50% methanol-water solution, centrifuged at 15,500 rpm for 15 min and injected 10 µl of the supernatant was injected to UPLC-MS/MS system for

quantification. The density of the blood is treated as 1g/mL in the tissue distribution study.

3.3.5.5 Preparation of standards, quality controls and sample solutions

Calibration standards and quality control (QC) samples were prepared as described in section 3.3.4.1.

3.3.5.6 Pharmacokinetic parameter calculation

The pharmacokinetic parameters of irinotecan, SN-38 and SN-38 glucuronide were calculated by the non-compartmental method, using Phoenix WinNonLin (Pharsight Corporation, Mountain View, California) program.

3.4. Results and Discussion

3.4.1. Optimization of the UPLC-MS/MS condition

Different combinations of mobile and stationary phases were employed to enhance the sensitivity of detection of irinotecan, SN-38 and SN-38 glucuronide. Acetonitrile, methanol, 0.1-5% formic acid in acetonitrile, and 0.1 - 5% formic acid in methanol as organic phase and 1 - 2.5 mM ammonium acetate in water, 0.05 - 1% formic acid in water as aqueous phase were tested as potential mobile phases. We tested both C8 and C18 column as stationary phases to resolve irinotecan, SN-38 and SN-38 glucuronide. Based on the intensity of the signal, signal-noise ratio and shape of the peak, 0.1% formic acid in water, 100% acetonitrile and C 18 column were found to be the optimal aqueous mobile phase, organic mobile phase and stationary phase, respectively. Apart

from that, it has been found that column temperature at 60˚C and the flow rate of 0.45 ml/min produced sharp and symmetrical peaks (Fig 4).

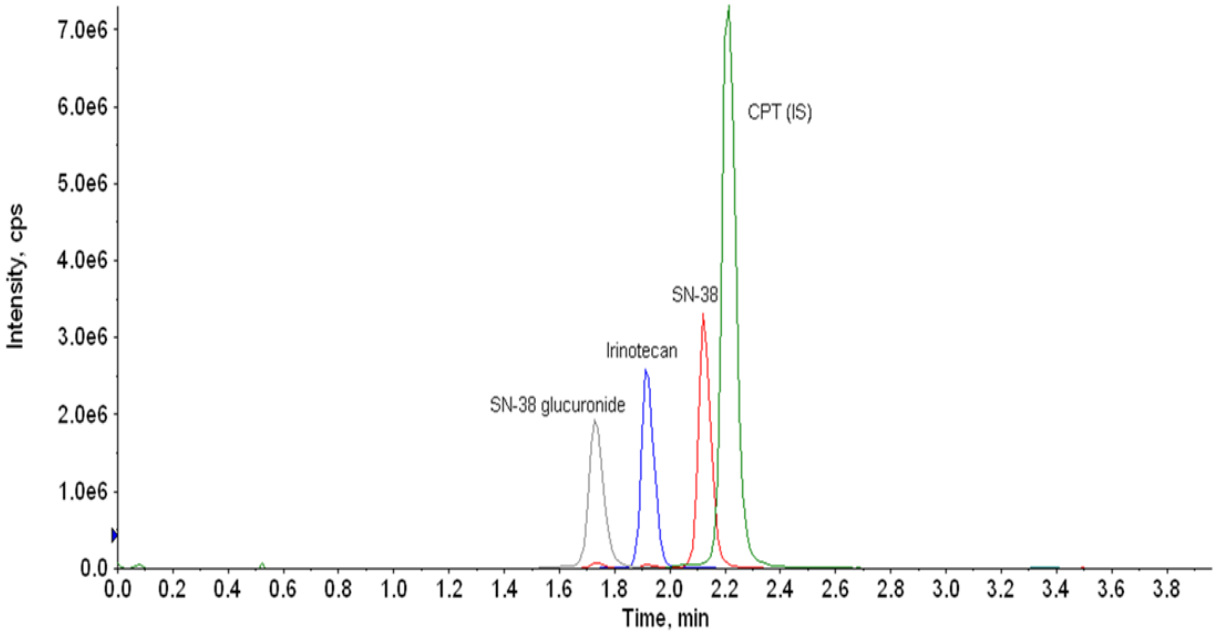


Figure 4: Representative UPLC chromatogram of irinotecan, SN-38, SN-38 glucuronide and CPT (IS).

For MS/MS analysis, both positive and negative scan mode were employed to analyze irinotecan, SN-38 and SN-38 glucuronide. A representative MS/MS spectrum of irinotecan, SN-38 and SN-38 glucuronide is shown in figure 5, 6 and 7, respectively. Based on the intensity of the analytes, positive scan mode was found to be more sensitive compared to the negative scan mode. To improve the specificity, multiple

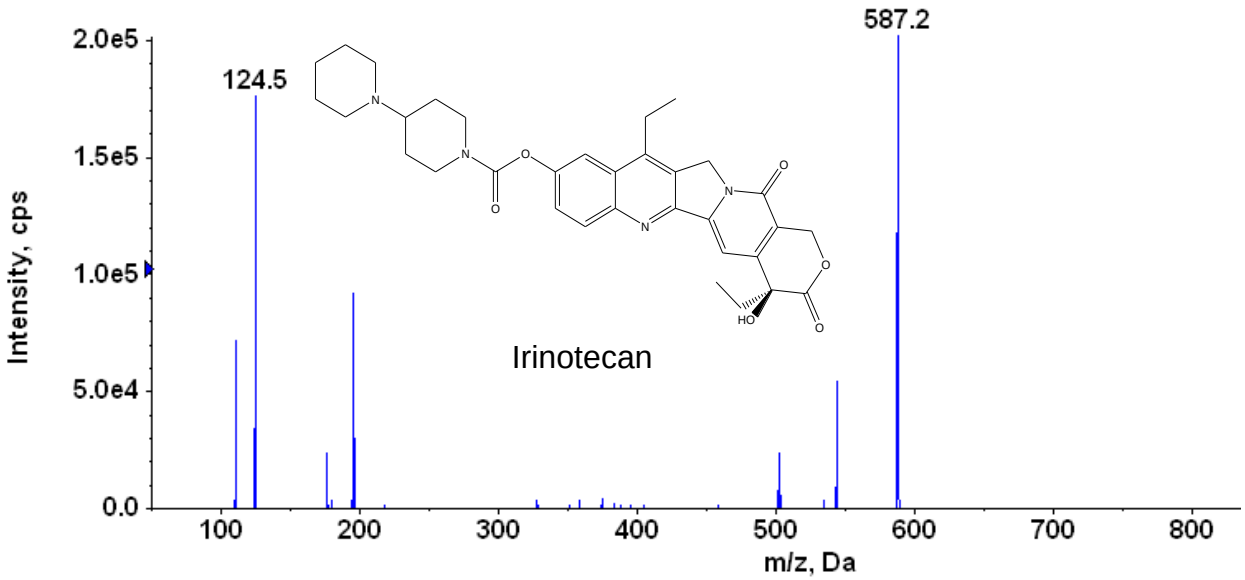


Figure 5: Chemical structures and representative MS/MS Chromatogram of irinotecan

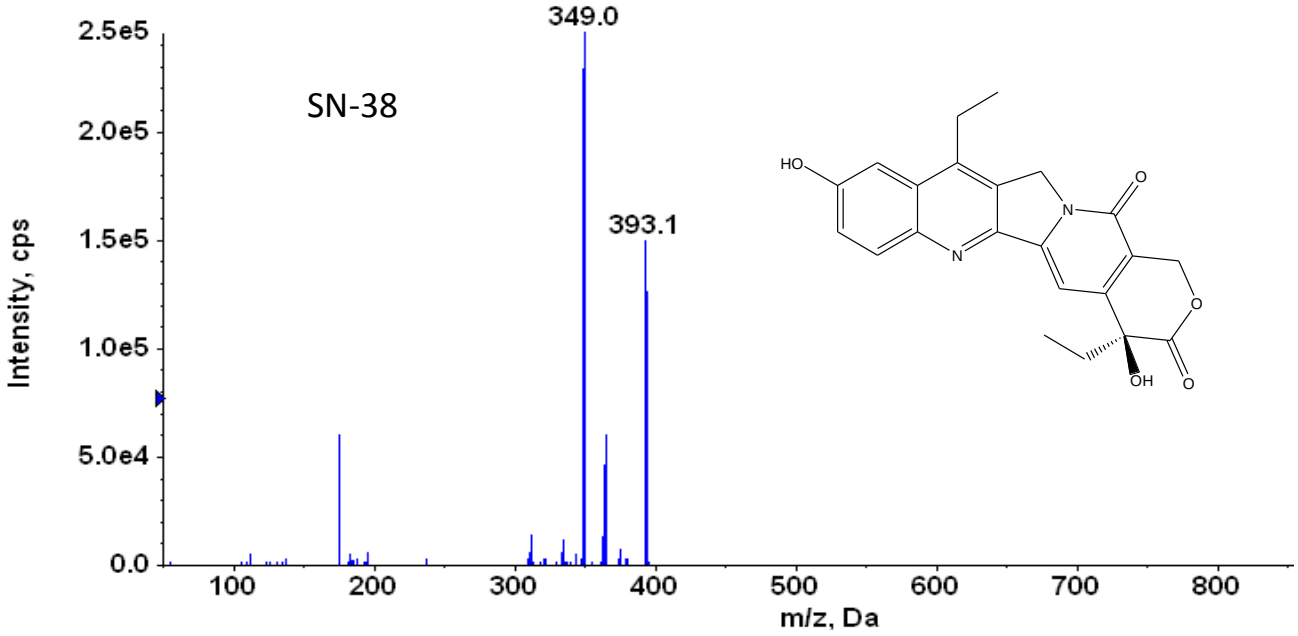


Figure 6: Chemical structures and representative MS/MS Chromatogram of SN-38

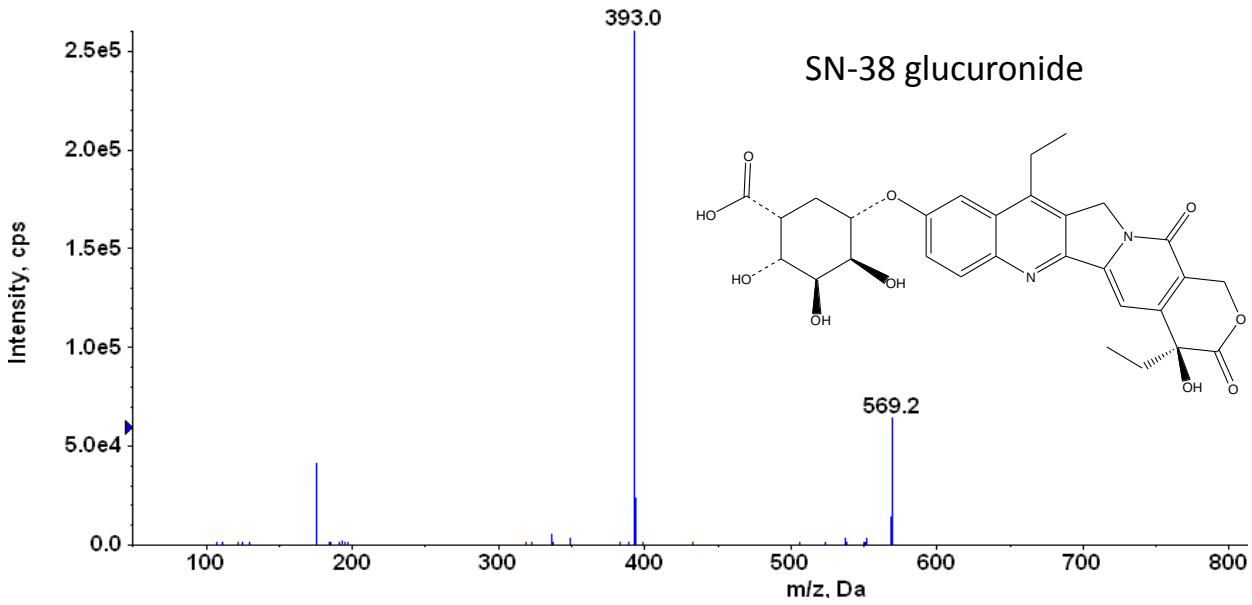


Figure 7: Chemical structures and representative MS/MS Chromatogram of SN-38 glucuronide

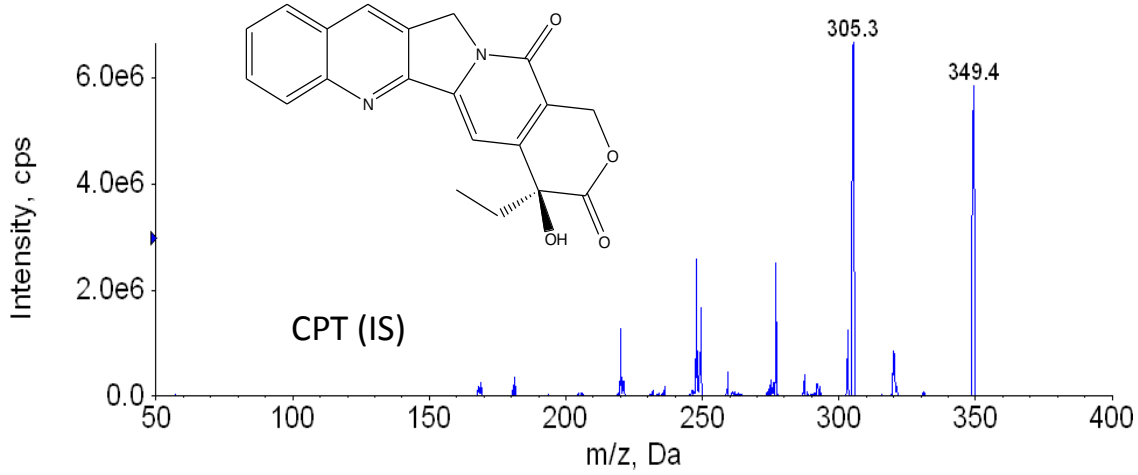


Figure 8: Chemical structures and representative MS/MS Chromatogram of CPT (IS)

Table 3: Compound dependent parameters used in UPLC-MS/MS Studies:

Analyte	Q1 (m/z)	Q3 (m/z)	Time (msec)	DP (Volts)	EP (Volts)	CE (Volts)	CXP (Volts)
CPT-11	587	124	100	161	10	43	8
SN-38	393	349	100	196	10	35	20
SN-38G	569	393	100	201	10	37	24
CPT	349	305	100	201	10	31	14

reactions monitoring (MRM) scan type was used. The MRM transitions from precursor ions to product ions were optimized as m/z 587.6 → 124.04 for irinotecan, 393.1 → 349.06 for SN-38, 569.05 → 393 for SN-38 glucuronide and 349 → 305 for CPT (IS), based on their most abundant precursor ions and corresponding product ions (Fig. 5, 6, 7 and 8). Additional compound-dependent parameters in MRM mode for irinotecan, SN-38, SN-38 glucuronide and CPT were summarized in Table 3. The main working parameters for mass spectrum were used in the QTRAP 5500 system as follows: ion-spray voltage, 5.5 kV; temperature, 500°C; curtain gas, 20 psig; gas 1, 20 psig; gas 2; 20 psig, collision gas, medium.

3.4.2. Method validation

3.4.2.1 Linearity and sensitivity

Method validation was conducted using blank rat plasma, untreated feces, liver and kidney tissue samples. In plasma, the standard curves of irinotecan, SN-38 and SN-38 glucuronide were linear in the concentration range of 4.88 - 10000 nM, 4.88 - 10000 nM, and 6.25 - 2000 nM SN-38 glucuronide, respectively (R^2 > 0.99). In case of feces samples, the standard curves of irinotecan, SN-38, SN-38 glucuronide were linear in the concentration range of 39 - 5000 nM, 39- 5000 nM, and 4.88 - 1250 nM SN-38 glucuronide, respectively (R^2 > 0.99). The assay also exhibited excellent linear response over selected concentration range of 48.8 – 6250 nM, 48.8 – 6250 nM and 9.8 – 1250 nM for irinotecan, SN-38 and SN-38 glucuronide, respectively in liver and kidney homogenates (R^2 > 0.99). The lower limit of detection (LLOD) of irinotecan and SN-38 was determined to be 2.44 nM, 19.5 nM and 24.44 nM in plasma, feces, liver and kidney homogenate, respectively. In case of SN-38 glucuronide, LLOD is 6.25 nM, 2.44 nM and 4.9 nM in plasma, feces, liver and kidney homogenate, respectively.

3.4.2.2 Accuracy and precision

Accuracy, intra-day and inter-day precision were determined by running six replicates of QC samples at three different concentration levels (low, medium, high) of irinotecan, SN-38 and SN-38 glucuronide in blank rat plasma, feces, liver and kidney tissues. The precision and accuracy of these measurements were shown in Table 4. These results demonstrated that the precision and accuracy values were in the acceptance range (< 15%) according to FDA guidance.

3.4.2.3 Recovery, matrix effect and stability

The mean extraction recoveries determined using three replicates of QC samples at three concentration levels (the same concentrations as QC sample) in rat plasma, feces, liver and kidney tissues were shown in Table 2, 3, 4 and 5, respectively. 50% methanol-acetonitrile and 100% acetonitrile were used as protein precipitating solvent to extract irinotecan, SN-38 and SN-38 glucuronide from blank rat plasma, liver, kidney and feces. The result showed that the recoveries were not less than 85% for all the analytes at three different concentrations in plasma and feces.

Similarly, to test if the matrix effects have any impact on the UPLC-MS analysis of irinotecan, SN-38 and SN-38 glucuronide, the relative peak areas of the analyte spiking in the evaporated blank plasma, feces, liver and kidney tissues at three different concentration levels (low, medium and high) were compared to similarly prepared standard solutions. As shown in the table 4, 5, 6 and 7, no measurable matrix effect was observed.

Stabilities of irinotecan, SN-38 and SN-38 glucuronide in plasma were evaluated by analyzing triplicates of QC samples at three different concentrations following storage at 25˚C for 4 h, at −80˚C for 3 days, and after going through three freeze–thaw cycles (−80˚C and 25˚C). All the samples showed 85–115% recoveries after various stability tests.

Table 4: Extraction recovery, intra-day and inter-day precision, and matrix effects for irinotecan, SN-38 and SN-38 glucuronide in plasma using MRM method (n=6)

Compound	Conc. (nM)	Extraction recovery	Intra-day		Inter-day		Matrix effect
		Average ± SD (%)	Accuracy (bias, %)	Precision (CV, %)	Accuracy (bias, %)	Precision (CV, %)	Average ± SD (%)
CPT-11	40	106.9 ± 4.45	104.34	13.19	103.48	6.04	85.67 ± 3.78
	200	100.44 ± 0.43	108.93	9.28	107.03	8.45	86.56 ± 3.81
	1000	109.06 ± 3.82	107.74	3.90	106.8	7.14	88.61 ± 0.50
SN-38	40	114.31 ± 13.28	110.35	5.44	85.98	1.80	92.79 ± 3.15
	200	108.5 ± 16.96	86.01	4.21	84.85	4.84	90.16 ± 2.01
	1000	102.34 ± 5.21	92.94	3.38	82.51	4.15	94.59 ± 1.03
SN-38G	8	99.12 ± 2.50	103.78	12.25	98.9	7.94	91.03 ± 1.66
	40	95.08 ± 0.44	105.90	3.11	93.73	5.21	90.42 ± 2.44
	200	99.52 ± 1.49	94.36	2.48	90.88	3.15	103.7 ± 0.81

Table 5: Extraction recovery, intra-day and inter-day precision, and matrix effects for irinotecan, SN-38 and SN-38 glucuronide in liver homogenate using MRM method (n=6)

Compound	Conc. (nM)	Extraction recovery Average ± SD (%)	Intra-day		Inter-day		Matrix effect Average ± SD (%)
			Accuracy (bias, %)	Precision (CV, %)	Accuracy (bias, %)	Precision (CV, %)	
CPT-11	40	92.09 ± 8.11	93.87	2.89	91.01	0.22	101.01 ± 12
	200	83.94 ± 5.65	95.04	5.97	103.50	0.13	95.97 ± 10.9
	1000	82.26 ± 4.38	91.55	2.66	107.77	0.94	96.3 ± 3
SN-38	40	80.28 ± 0.92	92.21	6.58	97.49	0.23	96.16 ± 5.4
	200	80.58 ± 3.64	95.39	3.46	97.94	0.34	84.23 ± 3.13
	1000	83.88 ± 5.98	96.75	3.74	97.53	2.19	95 ± 9.18
SN-38G	8	111.56 ± 4.75	96.04	2.75	108.4	0.01	91.96 ± 10.55
	40	95.51 ± 1.05	87.46	4.18	98.32	0.08	84.85 ± 3.95
	200	84.9 ± 3.62	95.16	3.26	98.43	0.42	97.41 ± 0.98

Table 6: Extraction recovery, intra-day and inter-day precision, and matrix effects for irinotecan, SN-38 and SN-38 glucuronide in kidney homogenate using MRM method (n=6)

Compound	Conc. (nM)	Extraction recovery	Intra-day		Inter-day		Matrix effect
		Average ± SD (%)	Accuracy (bias, %)	Precision (CV, %)	Accuracy (bias, %)	Precision (CV, %)	Average ± SD (%)
CPT-11	40	75.38 ± 4.94	91.79	8.62	98	0.06	111.25 ± 6.10
	200	79.15 ± 3.24	93.84	5.01	105.84	0.38	111.21 ± 5.50
	1000	77.82 ± 5.37	97.16	2.02	100.88	1.21	112.38 ± 3.90
SN-38	40	74.25 ± 6.23	95.32	5.79	100.07	0.06	97.17 ± 3.39
	200	77.67 ± 7.32	95.55	2.15	108.42	4.84	86.24 ± 3.55
	1000	78.96 ± 4.07	93.15	2.84	102.04	1.45	88.61 ± 0.50
SN-38G	8	86.83 ± 1.82	90.13	3.59	98.82	0.02	86.63 ± 2.95
	40	81.88 ± 6.09	98.95	0.98	109	0.07	90.88 ± 2.90
	200	83.77 ± 9.95	94.67	2.15	97.41	0.60	84.66 ± 6.21

Table 7: Extraction recovery, intra-day and inter-day precision, and matrix effects for irinotecan, SN-38 and SN-38 glucuronide in fecal homogenate using MRM method (n=6)

Compound	Conc (nM)	Extraction recovery	Intra-day		Inter-day		Matrix effect
		Average ± SD (%)	Accuracy (bias, %)	Precision (CV, %)	Accuracy (bias, %)	Precision (CV, %)	Average ± SD (%)
CPT-11	40	107.40 ± 8.95	90.84	2.9	81.60	1.13	94.75 ± 5.05
	200	109.14 ± 8.45	97.22	2.33	95.86	3.53	91.86 ± 4.07
	1000	107.07 ± 1.77	97.63	0.15	81.70	2.95	85.8 ± 6.39
SN-38	40	107.02 ± 2.68	96.66	1.69	80.29	3.61	88.26 ± 2.79
	200	106.9 ± 4.51	98.97	0.94	99.43	8.88	92.31 ± 2.84
	1000	102.49 ± 2.64	93.65	2.98	89.46	4.78	115.4 ± 8.43
SN-38G	8	83.8 ± 13.56	103.78	12.25	84.09	7.17	81.17 ± 4.31
	40	93.87 ± 16.84	105.9	3.11	100.45	0.39	81.92 ± 6.08
	200	86.63 ± 16.83	94.36	2.48	106.97	9.10	97.84 ± 1.4

.

3.4.3. Application to the method to determine the plasma, urine and feces concentration of Irinotecan in rats after i.v. administration

The validated method was utilized to determine irinotecan, SN-38 and SN-38 glucuronide content in plasma, feces and urine after intravenous administration of 5 mg/kg irinotecan in male wistar rats. Mean plasma concentration of irinotecan, SN-38 and SN-38 glucuronide as a function of time after i.v. administration of irinotecan is shown in fig 9. The C_{max} of irinotecan, SN-38 and SN-38 glucuronide are 6.02 ± 0.19 µM, 2.13 ± 0.70 µM and 0.08 ± 0.01 µM, respectively; whereas the T_{max} and AUC of irinotecan, SN-38 and SN-38 glucuronide are 33.75 min, 26.25 min, 100 min and 15.72 h µM, 18.53 h µM and 0.70 h µM, respectively. In case of urinary excretion it was found that in comparison to SN-38 (16.50 µM) and its glucuronidated form (11.47 µM), irinotecan (67.49 µM) predominantly excreted in unchanged form after 4 hours (fig 10). However, after 24 hours, the concentration of irinotecan (6.43 µM) and SN-38 glucuronide (6.07 µM) in urine is quite comparable. After analyzing the feces we found that, after 24 hour (fig 11), fecal content of irinotecan (818.35 µg/g) and SN-38 (423.95 µg/g) is quite higher in comparison to the fecal content of SN-38 glucuronide (9.60 µg/g). Low fecal excretion of SN-38 glucuronide might be ascribed to the hydrolysis of SN-38 glucuronide by bacterial β-glucuronidase in feces which was also supported by other reports (Sparreboom, de Jonge et al. 1998; Slatter, Schaaf et al. 2000).

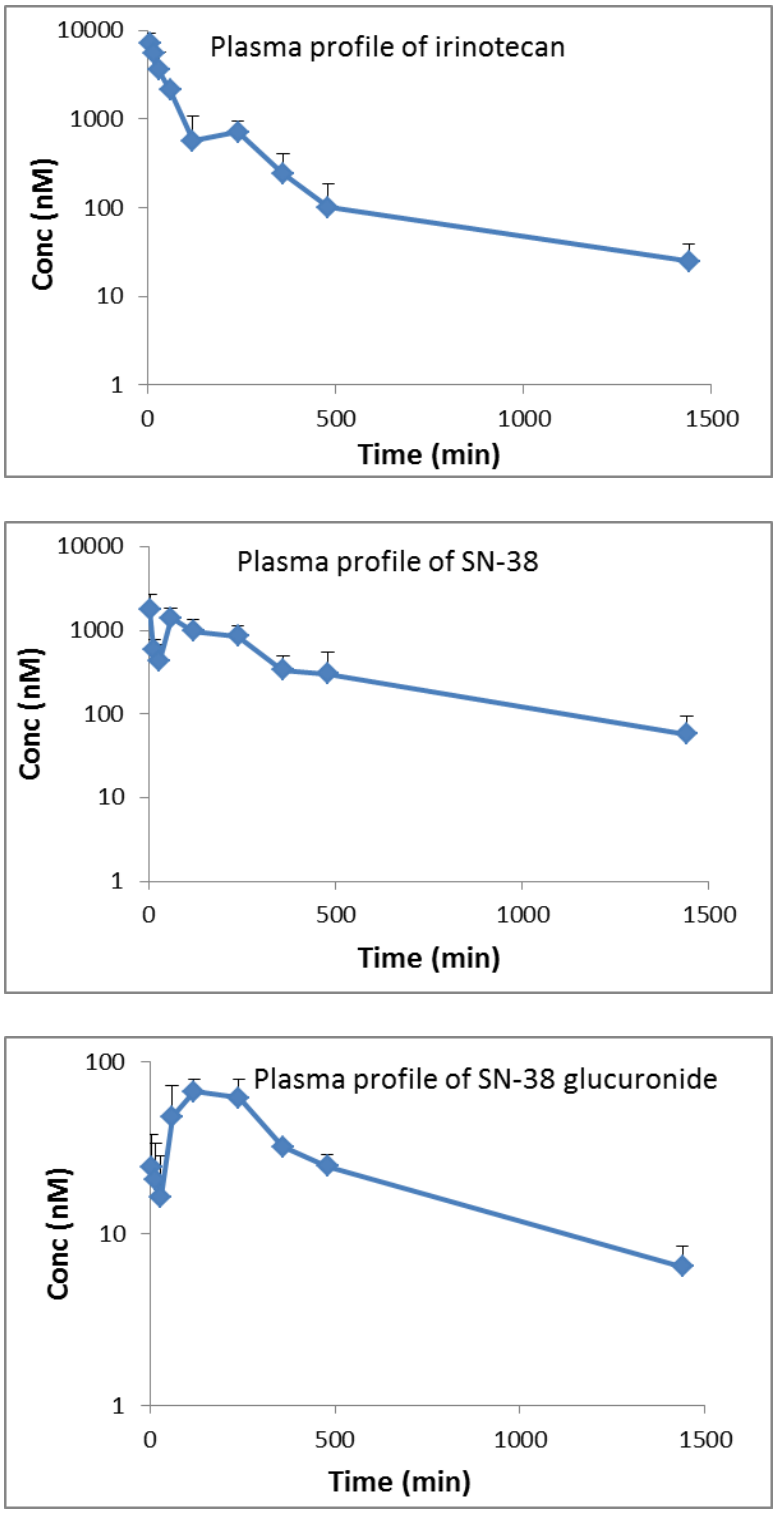
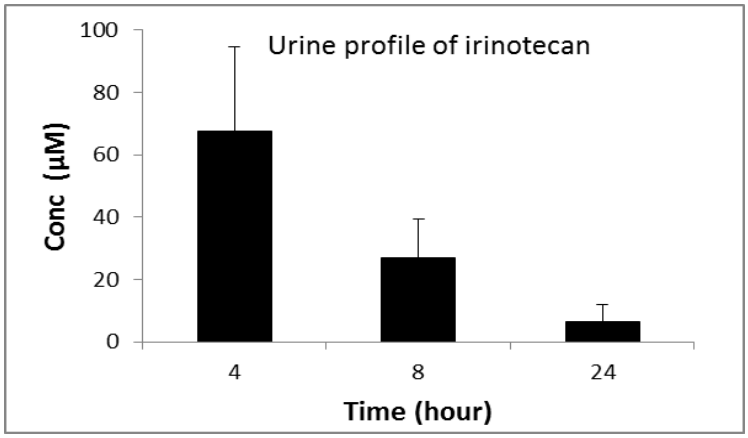
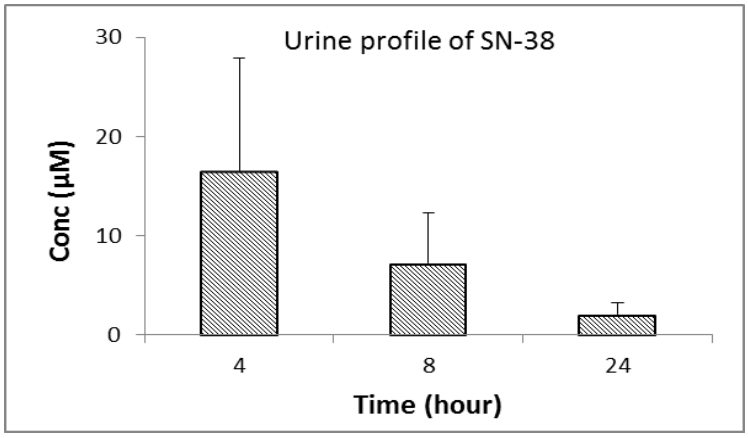


Figure 9: Plasma profile of irinotecan, SN-38 and SN-38 glucuronide after i.v. administration of 5 mg/kg irinotecan (n=4)





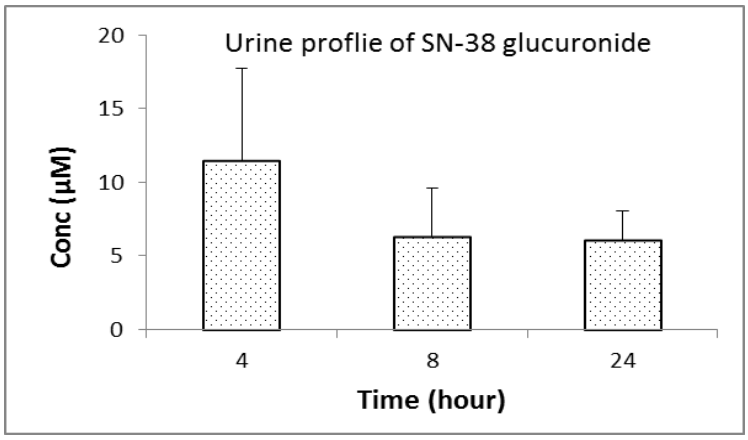


Figure 10: Urine profile of irinotecan, SN-38 and SN-38 glucuronide after i.v. administration of 5 mg/kg irinotecan (n=4)

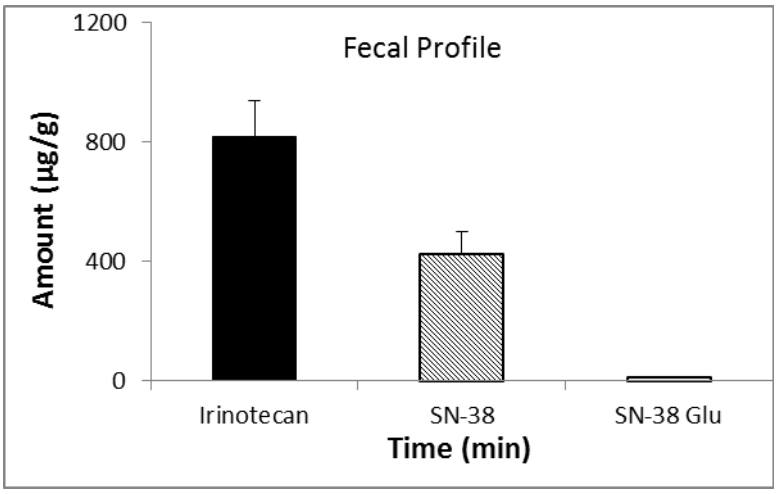


Figure 11: Fecal profile of irinotecan, SN-38 and SN-38 glucuronide after i.v. administration of 5 mg/kg irinotecan (n=4)

3.5. Conclusion

In this study, we developed and validated a rapid, sensitive and specific UPLC-MS/MS method for quantifying irinotecan, SN-38 and SN-38 glucuronide simultaneously in rat plasma, feces, urine, liver, kidney, and the validated method was successfully applied to the *in vivo* pharmacokinetic studies of irinotecan in rats. This is the first UPLC-MS/MS method that allows us to simultaneously quantify irinotecan, its active metabolite SN-38 and SN-38-glucuronide in different bio-matrices (plasma, urine and feces) as well as in different tissues (liver and kidney). Apart from that, this method has certain advantages such as high sensitivity (nM), short analysis time (4.5 min), good recovery with negligible matrix effect. This method can also be extrapolated to clinical studies owing to its high sensitivity and small sample volume requirement.

Chapter 4 Development of a Modified *in situ* Rat Intestinal Perfusion Model to simultaneously determine the disposition of irinotecan via Biliary, Intestinal and Urinary route

4.1. Abstract

The objective of this study was to develop a modified *in situ* rat intestinal perfusion model which can describe a holistic picture of the disposition pattern of irinotecan, its active metabolite SN-38 and SN-38 glucuronide (phase II metabolite of SN-38) in different metabolic organs (liver, intestine and kidney) as well as in different bio-matrices (bile, intestine, urine and plasma) simultaneously. For this purpose at first we performed a dose response study using our modified *in situ* rat perfusion model after intravenous infusion of three different doses of irinotecan (0.5, 5 and 50 mg/kg) for 150 minutes and collected bile, intestinal perfusate, urine and plasma at specific time period (30 min). Dose response study revealed that irinotecan and its metabolites predominantly excreted through bile (45 to 60%) in comparison to urine (4 to 15%) and intestinal secretion (3 to 10%); whereas irinotecan excreted predominantly as unchanged form (46 to 60%) compared to its active metabolite SN-38 (1.25 to 6.3%) and its conjugate form SN-38 glucuronide (2.1 to 10.5%) in all the bio-matrices irrespective of dose. Caco-2 cellular transport study of irinotecan and SN-38 reported the efflux ratio of irinotecan and SN-38 was 15.1 and 4.4, respectively which indicated the involvement of efflux transporter on the transport of irinotecan and SN-38. In addition, we investigated systematically the impact of major efflux transporters on the disposition of irinotecan and its metabolites at different organs using our modified *in situ* rat perfusion model by co-

administering different chemical inhibitors of efflux transporters (P-gp, MRP2, BCRP and MATE-1) with 5 mg/kg iv irinotecan. Chemical inhibition study demonstrated the possible involvement of MATE-1 transporter alongwith P-gp and MRP2 on the biliary and renal excretion of irinotecan. In our opinion, our *in situ* modified rat perfusion model may be regarded as a useful screening tool which could be utilized in drug-drug interaction or herb-drug interaction study as well as to elucidate a holistic feature of the disposition of drug candidate and xenobiotics.

4.2. Introduction

Irinotecan (CPT-11), a water soluble prodrug of SN-38, is a highly effective anticancer drug which is widely utilized in first and second line treatment of metastatic colon cancers typically in combination with other agents such as 5-flurouracil, leucovorin etc. (Saltz, Cox et al. 2000; Mathijssen, van Alphen et al. 2001). In addition, it also demonstrated clinical efficacy against other types of cancer such as lung, cervical, ovarian, gastric, refractory lymphoma and leukemia etc. (Ohno, Okada et al. 1990; Fukuoka, Niitani et al. 1992; Rosen 1998; Prados, Yung et al. 2004). Irinotecan is a semisynthetic derivative of a compound named camptothecin which is isolated from the bark of *Camptotheca acuminate* in 1966 (Wall M. E. 1966). The cytotoxic action of irinotecan is ascribed to its active metabolite SN-38, which is 100 to 1000 times more potent in comparison to irinotecan (Mathijssen, van Alphen et al. 2001). Irinotecan and SN-38, both shows cytotoxic action after forming an irreversible complex with DNA topoisomerase I enzyme and ligated double stranded DNA which ultimately causes cell death because of breaking the double stranded DNA (Redinbo, Stewart et al. 1998).

However, the clinical usage of irinotecan is often dose limited as it exhibits severe side effects such as late onset diarrhea and neutropenia (Armand 1996). Among these, neutropenia is often regarded as a common side effect of irinotecan chemotherapy and it can be treated by growth factors (G-CSF) (Hecht, Pillai et al. 2010). On the contrary, late onset diarrhea is considered to be a life threatening side effect which is observed in approximately 30 - 40% of the patient population particularly with high dose (Bleiberg and Cvitkovic 1996; Merrouche, Extra et al. 1997).

After intravenous administration, the carbamate-linked dipiperidino group of irinotecan cleaves to SN-38 through the action of liver carboxylesterases (mainly CES2) and subsequently SN-38 undergoes glucuronidation by Uridine 5'-diphospho-glucuronosyltransferase enzymes (UGT1A1) to form inactive SN-38 glucuronide to be excreted via bile to the intestines, where it can be hydrolyzed back to SN-38 by the action of bacterial β-glucuronidase secreted from commensal microbiota in colon (Ma and McLeod 2003; Smith, Figg et al. 2006). Efflux transporters (P-gp and MRP2) mediate the transportation of irinotecan, SN-38 and SN-38 glucuronide from systemic circulation to the bile and intestine in combination (Chu, Kato et al. 1997; Sugiyama, Kato et al. 1998; Smith, Figg et al. 2006). Different mechanistic studies hypothesized that the intestinal toxicity of irinotecan is associated with the intestinal accumulation of the active metabolite SN-38 caused by various mechanisms such as higher biliary excretion of SN-38 and SN-38 glucuronide mediated through different efflux transporters, deglucuronidation of SN-38 glucuronide to SN-38 by bacterial β-glucuronidase secreted by commensal microbiota, conversion of irinotecan to SN-38 in

gut by intestinal CES enzymes and enterohepatic recycling of irinotecan and SN-38 (Gupta, Lestingi et al. 1994; Catimel, Chabot et al. 1995; Takasuna, Hagiwara et al. 1996; Ahmed, Vyas et al. 1999; Khanna, Morton et al. 2000). As it is pretty evident that both phase II drug metabolizing enzymes as well as efflux transporters have a significant role in the intestinal toxicity of irinotecan, so modulation of those enzymes and efflux pumps might be considered as a viable option to ameliorate late onset diarrhea caused by irinotecan by decreasing colonic content of SN-38.

In situ rat intestinal perfusion or single pass intestinal perfusion is a FDA approved model which is widely utilized to characterize the absorption as well as disposition of drugs, nutrients and other xenobiotics according to the Biopharmaceutics Classification System (BCS). In general, compared to subcellular fractions (hepatic and intestinal microsome and S9) and cell systems (hepatocytes and enterocytes) *in situ* perfusion systems are always considered to be more physiological in nature and carries significant dynamic information. Compared to other models, the unique advantage of *in situ* intestinal perfusion model is that the blood supply to the perfused segment of the intestinal region is maintained throughout the experimental process which makes it a robust tool for simulating real *in vivo* conditions following oral drug administration (Hu and Amidon 1988; Hu, Roland et al. 1998). In addition, we can also utilize this particular model to determine the absorption of drug or drug candidate in the different regions of the intestine as the regional differences in the composition of the intestinal membrane and distribution of the uptake/efflux transporters as well as drug metabolizing enzymes are well documented. In our lab, an important extension of this model has been

employed where all four segments of the intestine (duodenum, jejunum, ileum and colon) were perfused simultaneously to monitor the effect of regional differences on the drug transport through intestine (Liu and Hu 2002). This particular extension of this method enables us to decrease the variability of the method as well as reduces the need to use a higher number of animals. In addition, our lab also modified the intestinal perfusion model to determine the contribution of the intestine vs liver in the excretion of phase II metabolites by introducing bile duct cannulation during the intestinal perfusion experiment (Chen, Lin et al. 2003). In that particular study, intestinal and biliary excretion of phase II conjugates of test drug were simultaneously measured to verify the relative metabolic contribution of the intestine versus the liver.

According to literature, both biliary and intestinal excretion of irinotecan are considered as important factors which contribute significantly in irinotecan induced intestinal toxicity (Araki, Ishikawa et al. 1993; Ahmed, Vyas et al. 1999; Khanna, Morton et al. 2000; Chen, Yueh et al. 2013). In this regard, simultaneous determination of biliary and intestinal excretion profile of irinotecan, SN-38 and SN-38 glucuronide will not only allow us to elucidate the relative contribution of biliary and intestinal excretion of irinotecan and its metabolites on the intestinal toxicity of irinotecan, but also enable us to explore different strategies to modulate contributing dispositional elements (drug metabolizing enzymes and efflux transporters) which will reduce the colonic content of SN-38 resulting in alleviation of the late onset diarrhea. Till date there are only a few studies available which explored both biliary and intestinal excretion of irinotecan, SN-38 and SN-38 glucuronide simultaneously (Arimori, Kuroki et al. 2001; Arimori, Kuroki et al.

2003). In this article, we developed a modified *in situ* rat perfusion model to simultaneously determine the biliary, intestinal and urinary excretion of irinotecan and its metabolites to observe/compare how irinotecan and its metabolites is preferentially eliminated through different excretion pathways (biliary, intestinal and urinary) at different doses. To the best of our knowledge, this is the first report where biliary, intestinal, urinary excretion as well as bio-distribution of irinotecan, SN-38 and SN-38 glucuronide in liver and kidney has been determined simultaneously. Unlike previous studies in our lab, this is the first time we cannulated the urinary bladder of the animal in the perfusion experiment to collect urine at the same time point as perfusate and bile. The purpose of introducing urinary cannulation in the perfusion experiment is to get a holistic picture (mass balance) about the excretion of irinotecan through different bio-matrices. In addition, we also performed a Caco-2 cell culture study to explore the pattern of absorption/efflux of irinotecan and SN-38. Finally, we conducted a systematic chemical inhibition study employing different chemical inhibitors of efflux transporters using our newly developed modified *in situ* rat perfusion model to assess the impact of efflux transporters on the disposition of irinotecan at different organs (liver, kidney and intestine).

4.3. Materials and methods

4.3.1. Materials

Irinotecan, SN-38, cyclosporine A, erlotinib, cimetidine, camptothecin (CPT), uridine-5'-diphosphate-β,D-glucuronic acid ester (UDPGA), D-saccharic-1,4-lactone monohydrate, magnesium chloride, hanks' balanced salt solution (powder form) and formic acid were

purchased from Sigma–Aldrich (St. Louis, MO, USA). Expressed human UGT isoforms (UGT1A1) was purchased from BD Biosciences (Woburn, MA, USA). Solid phase extraction (C18) columns were purchased from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile, methanol and water (LC-MS grade) were purchased from EMD (Gibbstown, NJ, USA). Water was deionized by a Milli-Q water purification system of Millipore (Bedford, MA, USA). Intravenous irinotecan hydrochloride injection (20 mg/ml) was purchased from Teva Pharmaceuticals (Pearl River, NY, USA). All other materials (typically analytical grade or better) were used without further purification. Cloned Caco-2 cells (TC7) were a kind gift from Dr. Monique Rousset (Institut National de la Sante et de la Recherche Medicale U178, Villejuit, France).

4.3.2. Biosynthesis of SN-38 glucuronide

SN-38 glucuronide was biosynthesized using human expressed UGTs (UGT1A1 isoform). In this study, SN-38 was incubated with UGT1A1 for 24 hours which resulted in the formation of95.8% SN-38 glucuronide. We separated the residual aglycone (SN-38) from the mixture after employing liquid-liquid extraction utilizing dichloromethane (DCM). Then each 5 ml of processed samples were applied to a C18 solid phase extraction column. After washing out the salt, 1 ml of methanol was used to elute the SN-38 glucuronide. The eluted fractions of methanol were collected and air dried, and the residue was reconstituted with 100 µl of 50% methanol-water to concentrate SN-38 glucuronide. In each step, we measured the respective peak area of SN-38 and SN-38 glucuronide in UPLC to calculate the purity of SN-38 glucuronide in the mixture. Apart

from UPLC quantification, we also utilized MS/MS spectrum of SN-38 glucuronide to confirm its presence in the solution.

4.3.3. Transport experiments in Caco-2 cell culture model

Cell cultures were prepared as described previously by our laboratory (Yang, Gao et al. 2010; Gao, Yang et al. 2011). Cells were used between passages 41- 49. Briefly, a cell monolayer was prepared by seeding 400,000 cells per insert (Nunc, surface area = 4.2 cm^2, 3 µm pore size). Cells were maintained at 37ºC less than 90% humidity and 5% CO_2. Monolayers were used between 19 and 22 days after seeding for Caco-2 cells. The integrity of each monolayer was checked by measuring the transepithelial electrical resistance (TEER; Millicell ERS) before the experiment. The normal TEER values obtained were above 500 $\Omega \cdot cm^2$ for Caco-2 cells. HBSS (9.8 g/mL) supplemented with $NaHCO_3$ (0.37 g/L), HEPES (5.96 g/L), and glucose (3.5 g/L) was used for all experiments after the pH had been adjusted to 7.4.

The experimental protocol and calculations were described in our previous reports (Yang, Gao et al. 2010; Gao, Yang et al. 2011). Briefly, 5 µM solution of irinotecan and SN-38 in HBSS buffer was loaded onto the apical or basolateral (donor) side. Five donor samples (200 µL) and five receiver samples (200 µL) were taken at 0, 1, 2, 3, and 4 h followed by the addition of 200 µL of fresh donor solution to the donor side or 200 µL or fresh buffer to the receiver side. In this experiment, 10 µM rutin was used as an internal standard in this experiment. The samples were then analyzed by UPLC-MS/MS. The apparent permeability coefficient (P) was determined by the equation,

$$P = \left(\frac{dQ}{dt}\right)/(A \times C0)$$

Where, dQ/dt is the drug permeation rate (µmol/s), A is the surface area of the epithelium (cm^2), and C_0 (mM) is the initial concentration in the donor compartment at time zero. We also calculated the efflux ratio value of irinotecan and SN-38 from the mean apical to basolateral (A-B) P_{app} data and basolateral to apical (B-A) P_{app} data.

$$\text{Efflux ratio} = (\text{Permeability}_{\text{B to A}}) / (\text{Permeability}_{\text{A to B}})$$

4.3.4. Animals

Male Wistar rats (6-10 weeks, Body weight between 280 to 320 g) were purchased from Harlan Laboratory (Indianapolis, IN). Rats were kept in an environmentally controlled room (temperature: 25 ± 2°C, humidity: 50 ± 5%, 12 h dark-light cycle) for at least 1 week before the experiments. The rats were fasted overnight before the day of the experiment.

4.3.5. Animal Surgery

The procedures were approved by the University of Houston's Institutional Animal Care and Uses Committee (IACUC). Figure 1 represents the schematic of the modified intestinal rat perfusion model which provides an overview of the animal model. Before starting the surgery, at first isoflurane-oxygen mixture was used to anaesthetize wistar rats and then 50% dose of urethane (2g/kg) was injected through intramuscular route. After injecting urethane, we waited for 30-45 minutes to inject the rest of the urethane to

make sure that the rat is not showing any kind of unusual behavior. We started to perform our surgery once we are certain that the rat is totally unconscious. At first, the rat was put on a heating blanket under a heating lamp to stabilize its normal body temperature. The skin of neck and abdomen was properly shaved for surgery and cannula insertion. For bile duct cannulation, a 5 cm abdominal midline incision is made to locate duodenum and then the fatty tissues are removed from the surrounding tissues to separate the bile duct from the surface. Next, we inserted a tubing (PE10) after making a small cut with a micro vascular scissor and secured the tubing with a surgical suture. To cannulate one segment of jejunum, we inserted an inlet cannula at the beginning of the jejunum whereas the outlet cannula was inserted about 20-25 cm below the inlet one. After suturing both of the cannulas, we put the intestine into the abdominal cavity which was covered by a saline-wetted cotton towel to prevent excessive bleeding and/or moisture loss. In addition, the inlet cannula was insulated and kept warm at 37°C by a circulating water bath to keep the temperature of the perfusate constant. For urinary bladder cannulation, first we located the top portion of the urinary bladder and make a small cut with a micro vascular scissor. Then we inserted a soft tubing (PE10) through the cut and secured the tubing with a surgical suture which will allow urine to flow into the collection vials. Lastly, for cannulation of jugular vein we made a 1.5 cm incision on the left neck. Then we removed the fatty tissue from the jugular vein surface after dissecting different layers of tissues that cover the vein (e.g., superficial fascia and shallow muscularis). After locating and identifying the jugular vein, we inserted a tubing (PE20) through a small incision made by a microvascular scissor to collect blood from jugular vein.

4.3.6. Intestinal perfusion experiment

This modified *in situ* rat perfusion experiment is a single-pass perfusion method. One segment of jejunum was perfused with a HBSS buffer using an infusion pump (model PHD 2000; Harvard Apparatus Inc., Holliston, MA) at a flow rate of 0.191 ml/min for 150 min. After a 30-min washout period, which was usually sufficient to achieve the steady-state absorption, four samples were collected from the outlet cannula periodically (every 30 min). Apart from perfusate samples, we also collected bile (0.4 ml), urine (0.2 ml), and blood samples (0.1 ml) at 30 minute interval. Blood samples were centrifuged at 5000 rpm for 5 min and the pellet was discarded. The outlet concentrations of irinotecan, SN-38 and SN-38 glucuronide in bile, intestinal perfusate and plasma were determined by UPLC-MS/MS; whereas all urine samples containing irinotecan and its metabolites were determined by UPLC. For chemical inhibition study, we infused cyclosporine A (4 mg/kg iv) and cimetidine (5 mg/kg iv) through jugular vein alongwith irinotecan, whereas quercetin (50 μM) and erlotinib (50 μM) perfused through the segment of jejunum after dissolving in HBSS buffer.

4.3.7. Tissue homogenization

Liver and kidney were excised from the sacrificed rats and stored frozen at −80°C in polypropylene tubes until homogenization. The frozen tissues were thawed, chopped and weighed at 4°C. Accurately 50 mg of chopped tissue was homogenized in 2 ml of ice-cold homogenizing solution (pH 7.4) containing 10 mM potassium phosphate, 250 mM sucrose and 1 mM EDTA dehydrate with a polytron tissue homogenizer. Homogenization was paused 20 s after every 30 s of homogenization at a medium

speed. The homogenization was repeated 3 – 4 times until a uniform homogenate was obtained. Final tissue extract was stored at approximately −80°C prior to analysis. The homogenizer probe was washed sequentially with water, methanol and water after every homogenization.

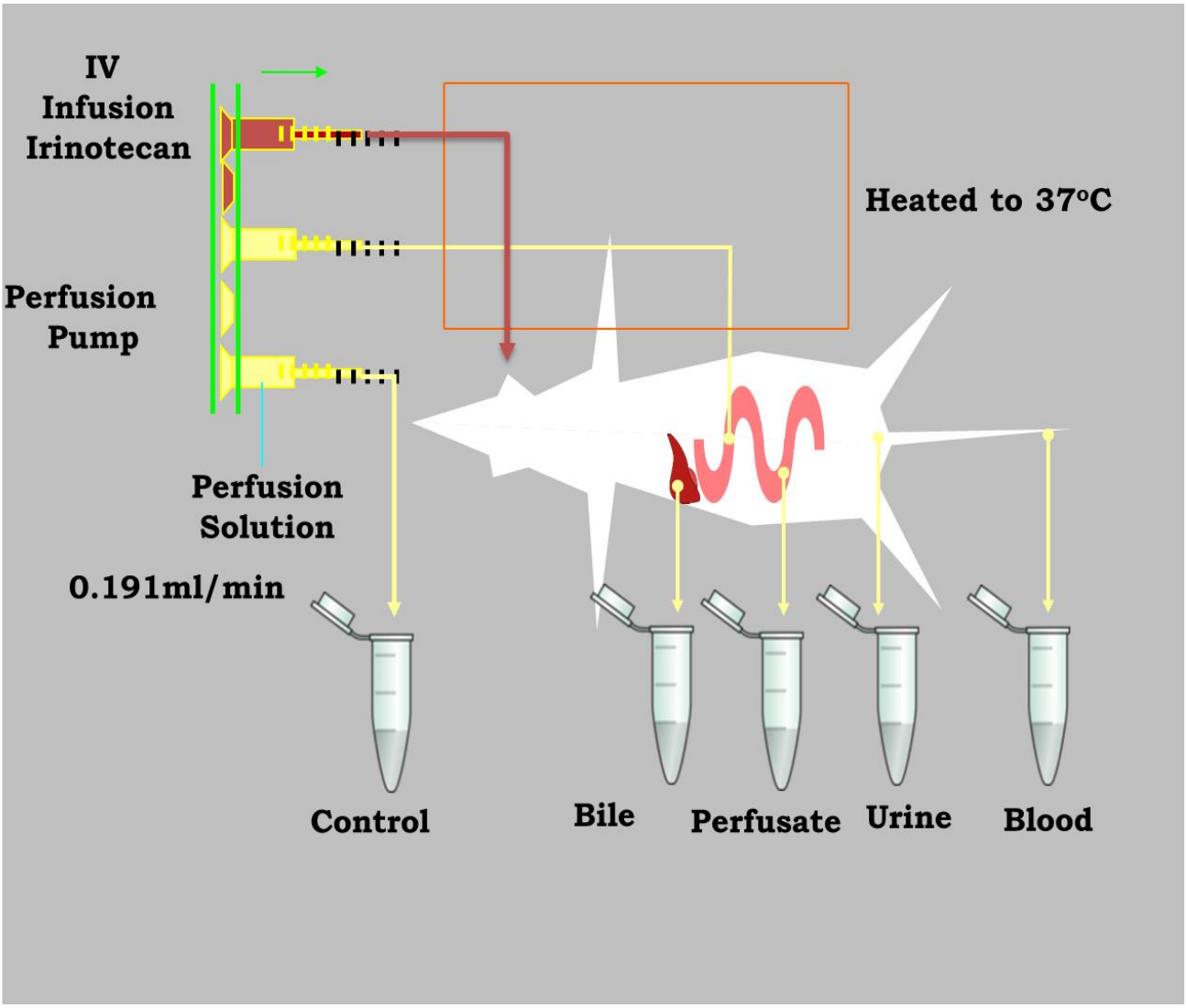


Figure 12: Schemetic diagram of the modified *in situ* rat perfusion model used with various sample points in the current study. Irinotecan was administered into the system at a constant rate (infusion at 5 ml/hr) and blank HBSS buffer (perfusion at 0.191 ml/min). Each sample interval was 30 min, and total operation time was 150 min to demonstrate the achievement of the steady-state.

4.3.8. Sample preparation

We diluted bile samples (5 µl) 100 times by 50% aqueous methanol solution and added 50 µl 0.1 µM CPT in 50% methanol-water as internal standard to the solution. In case of urine and perfusate samples, we diluted 10 and 50 µl samples with 100 and 50 µl 50% methanol-water, respectively and added 50 µl of testosterone in 94% acetonitrile-6% acetic acid mixture (internal standard) and 100 µl 0.1 µM CPT in 50% methanol-water (internal standard). After addition of 50% methanol-water solution and internal standard, all the processed samples (bile, urine and perfusate) were vortexed, centrifuged for 15 min at 15500 rpm and 10 µl supernatant was injected to UPLC-MS/MS and UPLC for analysis. Plasma, liver and kidney homogenate samples (20 µl) were spiked with 20 µl of internal standard (CPT in 50% methanol, 100 nM) and vortexed for 1 min after extracted with 50% methanol-acetonitrile solution in the ratio of 1:9:9 (solution: methanol: acetonitrile; v/v). All solutions were vortexed and centrifuged at 15,500 rpm for 15 min. The supernatants were transferred to another tube and evaporated to dryness under a steady stream of air at room temperature. The residue was reconstituted with 80 µl of 50% aqueous methanolic solution and centrifuged again at 15,500 rpm for 15 min. After centrifugation, 10 µl of the supernatant was injected to UPLC-MS/MS system for analysis. The density of the blood is treated as 1g/mL in the tissue distribution study.

4.3.9. UPLC-MS/MS Analysis of irinotecan, SN-38 and SN-38 glucuronide

The chromatographic separation of irinotecan, SN-38 and SN-38 glucuronide was achieved by a Waters Acquity™ UPLC H-Class system with a diode array detector (DAD) and a flow-through-needle sample manager. Analysis was carried out using

Acquity UPLC BEH C18 Column (2.1 mm × 50 mm, 300˚A, 1.7 µm, Waters, Milford, MA, USA). Mobile phase A (0.1% formic acid in water [v/v]) and mobile phase B (100% acetonitrile) were operated with a gradient elution at a flow rate of 0.4 ml/min as follows: 10% B → 25% B (0 – 0.5 min), 25% B → 40% B (0.5 - 1 min), 40% B (1 – 2.5 min), 40% B → 10% B (2.5 – 4.5 min). The column temperature and sample temperature was 60°C and 20°C. The injection volume was 10 µl. 100 nM camptothecin (CPT) in 50% methanol-water was used as internal standard (IS). LC-MS/MS analysis for irinotecan, SN-38 and SN-38 glucuronide was performed with an API 5500 Qtrap triple quadruple mass spectrometer coupled with a TurboIonSpray™ (Applied Biosystem- MDS SCIEX, Framingham, MA, USA). The system was operated in positive electrospray ionization (ESI) and multiple reactions monitoring (MRM) scan mode. All data were acquired and processed using Analyst®1.5.2 software with hotfixes (AB SCIEX). The main working parameters for mass spectrum were used in the QTRAP 5500 system as follows: ion-spray voltage, 5.5 kV; temperature, 500˚C; curtain gas, 20 psig; gas 1, 20 psig; gas 2; 20 psig, collision gas, medium. The quantification was performed using MRM method and compound-dependent parameters in MRM mode for irinotecan, SN-38, SN-38 glucuronide and CPT were summarized in Table 3.

4.3.10. Statistical test

One way ANOVA with dunnet's pothoc tests was used to analyze the data of plasma, liver and kidney accumulation profile of irinotecan, SN-38 and SN-38 glucuronide in dose response and chemical inhibition study. Two-way analysis of variance test with bonferoni posttest (Graphpad prism) was used to analyze the data of the biliary,

intestinal and urinary profile of irinotecan, SN-38 and SN-38 glucuronide in dose response and chemical inhibition study. The prior level of significance was set at 5%, or $p < 0.05$. In this article, '*'denotes p value less than 0.05, '**'denotes p value less than 0.01 and '***'denotes p value less than 0.001.

4.4. Results

4.4.1. Dose response study of disposition of irinotecan, SN-38 and SN-38 glucuronide using modified *in situ* rat perfusion model:

In this article we developed a modified *in situ* rat perfusion model which determined different bio-matrices such as bile, intestinal perfusate, urine and plasma at 30 min interval during the perfusion experiment. At first we performed dose response study of irinotecan disposition to demonstrate how different excretion pathways of irinotecan and its metabolites altered at different doses. In addition, we also determined the accumulation of irinotecan and SN-38 in two major metabolic organs (liver and kidney).

4.4.1.1 Biliary excretion rate and cumulative biliary excretion profile of irinotecan:

Biliary excretion rate (panel a to c) and cumulative biliary excretion (panel d to f) profile of irinotecan, SN-38 and SN-38 glucuronide at after iv administration of 0.5, 5 and 50 mg/kg of irinotecan through jugular vein for 150 min were represented in the figure 13. Biliary excretion rate profile (figure 13, panel a to c) of irinotecan, SN-38 and SN-38 glucuronide suggested that at all three different doses the biliary excretion of all three compounds reached steady state after 90 minutes of irinotecan administration. At 0.5 and 5 mg/kg dose, the biliary excretion rate of irinotecan is 7.5 -10 fold and 3.5 - 6 fold

higher than the biliary excretion rate of SN-38 and SN-38 glucuronide; whereas at higher dose the excretion rate of irinotecan accelerated more (approximately 40-50 fold) compared to SN-38 and SN-38 glucuronide. Cumulative biliary excretion profile (figure 13, panel d to e) of irinotecan, SN-38 and SN-38 glucuronide also reflected the same trend where at low and middle dose (0.5 and 5 mg/kg), the excretion of irinotecan through bile is 8-10 fold and 3-6 fold higher in comparison to SN-38 and SN-38 glucuronide. Similarly, at higher dose (50 mg/kg) predominant biliary excretion of irinotecan is observed compared to SN-38 and SN-38 glucuronide (20 to 35 fold higher).

4.4.1.2 Intestinal excretion rate and cumulative intestinal excretion profile of irinotecan:

Contrary to biliary excretion, the intestinal excretion rate profile (figure 14, panel a to c) indicates that excretion of irinotecan, SN-38 and SN-38 glucuronide reached steady state within 1 hour of starting the iv irinotecan infusion except irinotecan at 50 mg/kg dose (90 min). Intestinal excretion rate of SN-38 glucuronide wasn't significantly changed over 5 &50 mg/kg dose. The cumulative intestinal excretion profile (figure 14, panel d to e) demonstrated that at 5 and 50 mg/kg dose, the cumulative intestinal excretion of irinotecan relative to SN-38 and SN-38 glucuronide (100 – 130 fold) is quite higher in comparison to the biliary excretion (3 - 35 fold).

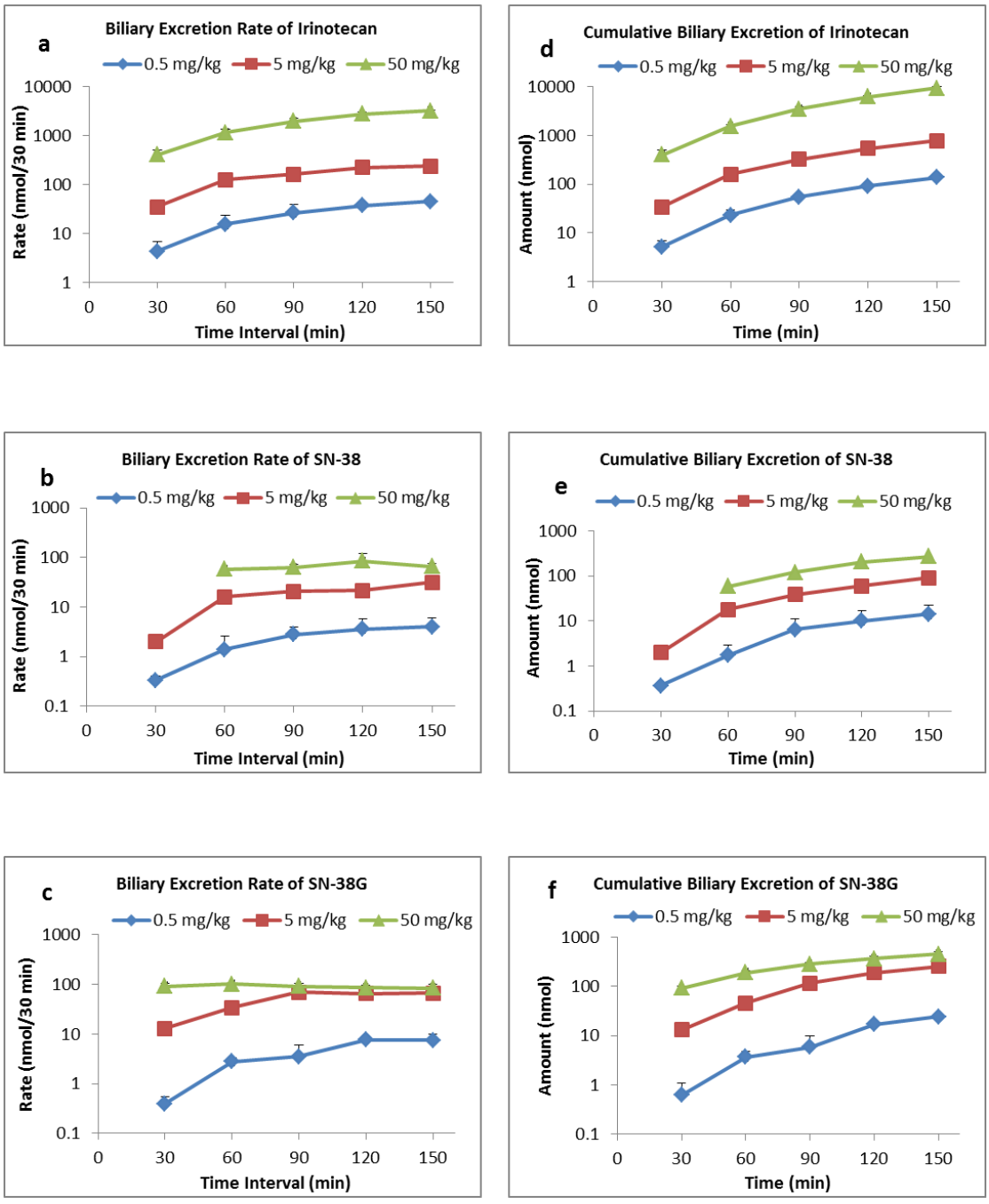


Figure 13: Biliary excretion rate profile and cumulative biliary excretion profile of irinotecan, SN-38 and SN-38 glucuronide at different dose of irinotecan (n=4).

4.4.1.3 Urinary excretion rate and cumulative urinary excretion profile of irinotecan:

One of the major advantages of this model is the simultaneous determination of the urinary excretion profile of irinotecan, SN-38 and SN-38 glucuronide alongwith the biliary and intestinal excretion at same point of time throughout the experiment. The urinary excretion rate profile (figure 15, a to c) denoted that the urinary excretion rate of SN-38 remained constant throughout different doses whereas urinary excretion rate of irinotecan and SN-38 glucuronide is dose dependent. Cumulative urinary excretion plots (figure 15, d to e) also indicated that irinotecan predominantly excreted through urine as unchanged form rather than its metabolite form SN-38 (10 to 80 fold) and SN-38 glucuronide (13 to 35 fold).

4.4.1.4 Plasma profile of irinotecan:

Apart from the biliary, intestinal and urinary excretion profile we also simultaneously determined the plasma profile of irinotecan as well as its metabolites to observe whether at different doses the systemic level of irinotecan and SN-38 remained dose dependent. Figure 16 described the plasma profile of irinotecan (panel a), SN-38 (panel b) and SN-38 glucuronide (panel c) which showed that at different doses the plasma concentration of all three compounds remained constant throughout the experiment after achieving steady state within 30 min. Table 3 described the AUC values of irinotecan, SN-38 and SN-38 glucuronide in dose response study.

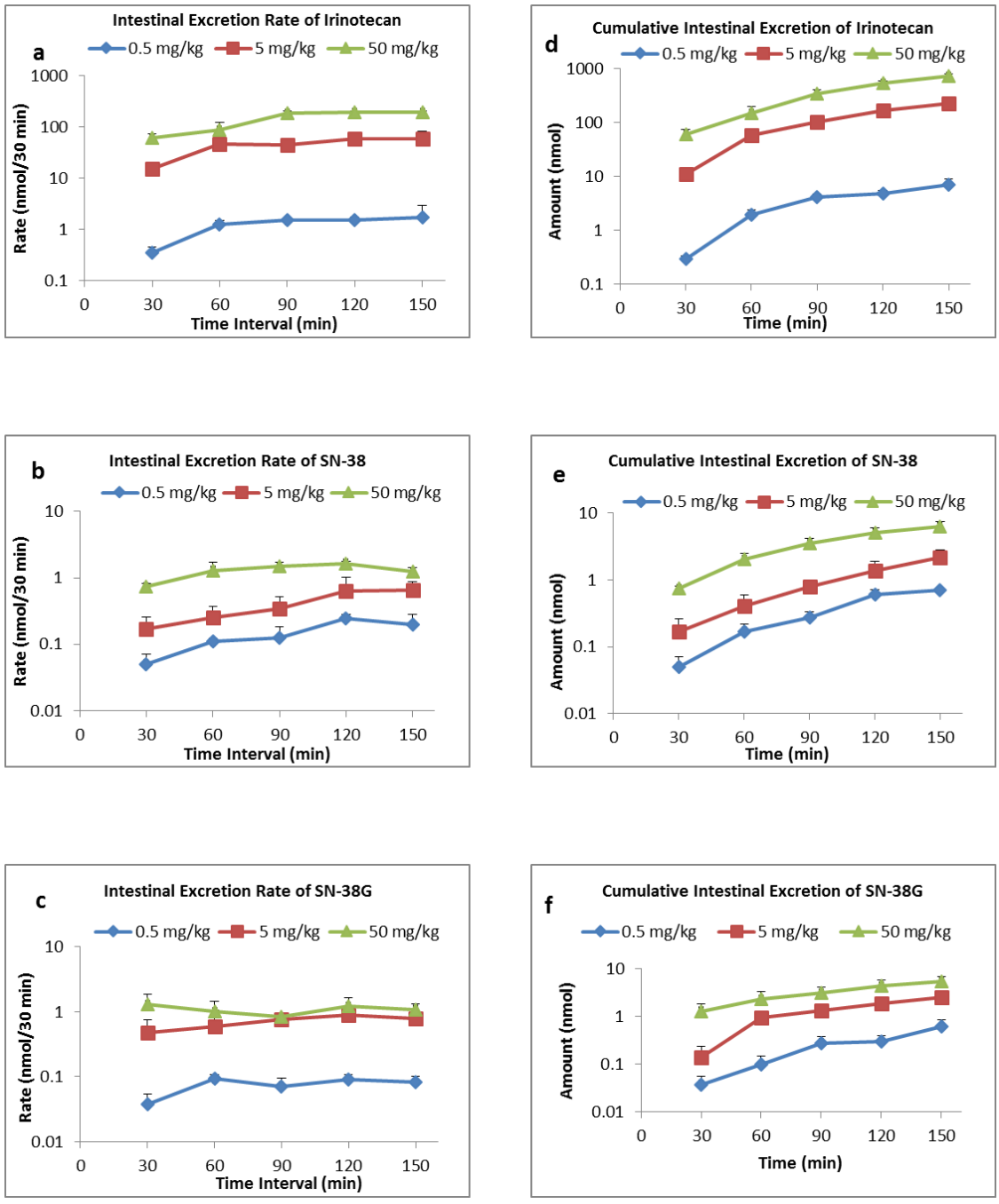


Figure 14: Intestinal excretion rate profile and cumulative intestinal excretion profile of irinotecan, SN-38 and SN-38 glucuronide at different dose of irinotecan (n=4).

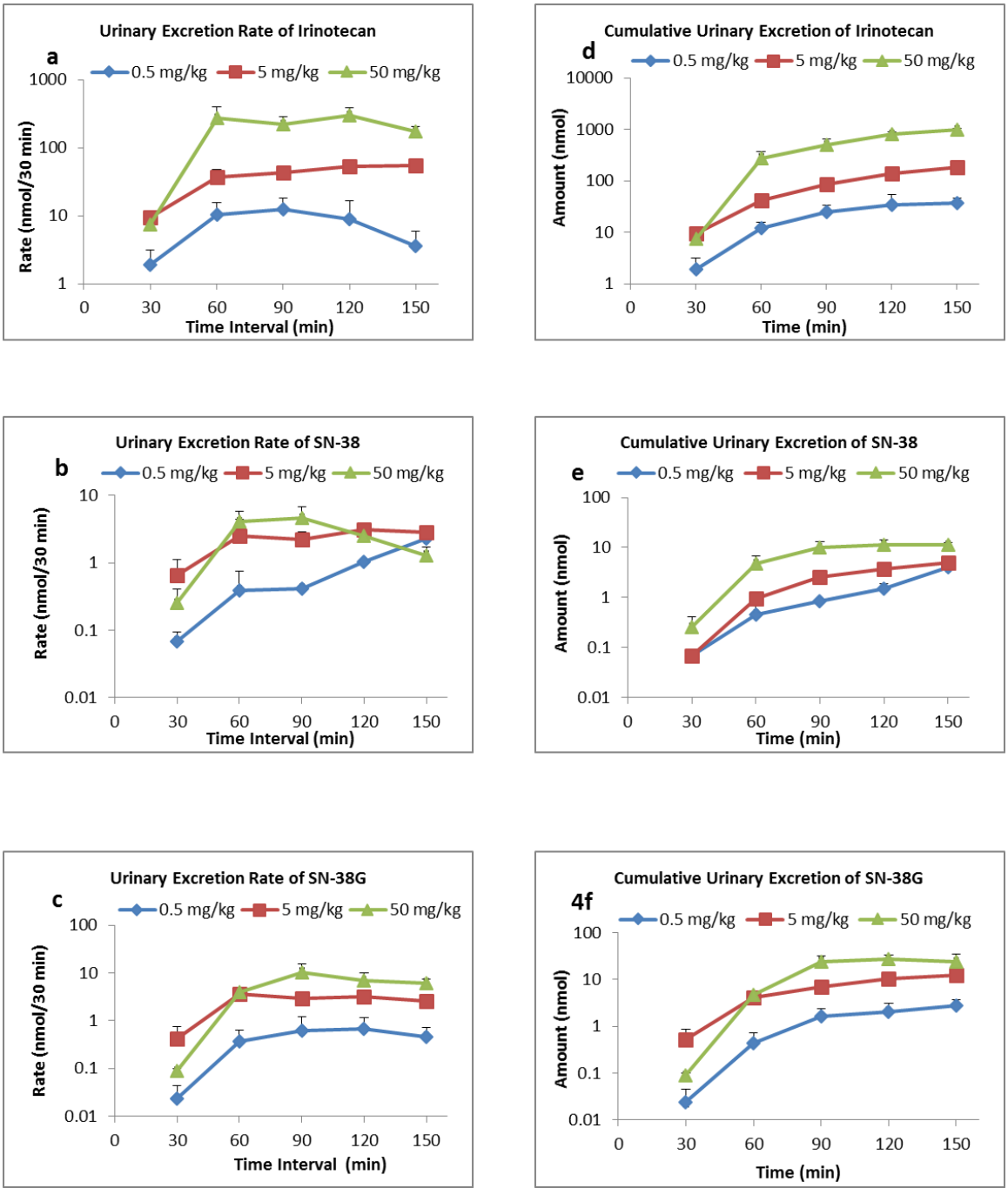


Figure 15: Urinary excretion rate profile and cumulative urinary excretion profile of irinotecan, SN-38 and SN-38 glucuronide at different dose of irinotecan (n=4).

4.4.1.5 Bio-distribution of irinotecan in liver and kidney:

Bio-distribution study of irinotecan in liver and kidney (figure 17) suggested that at higher dose (5 and 50 mg/kg) irinotecan (4 fold) and SN-38 (14 fold) preferentially distributed in liver compared to kidney, whereas accumulation of SN-38 glucuronide is higher in kidney (3 to 6 fold). In addition, figure 17 also indicates that there is no significant difference of accumulation of irinotecan, SN-38 and SN-38 glucuronide observed at 0.5 and 5 mg/kg, whereas at 50 mg/kg dose, all the chemicals accumulated at significant amount.

4.4.2. Irinotecan and SN-38 transport in Caco-2 cell culture model:

The Caco-2 cell culture model is well recognized and commonly used model of the human intestine. Owing to the easy access of both apical and basolateral sides of the intestinal epithelium, it is considered to be an excellent model for studying drug excretion or efflux. In this particular study, we conducted this experiment to determine the efflux ratio of irinotecan and SN-38. In Caco-2 cells, the absorption permeability (from apical to basolateral side, A to B) of irinotecan and SN-38 was $6.01 \pm 0.18 \times 10^{-7}$ cm/sec and $4.33 \pm 0.61 \times 10^{-6}$ cm/sec, respectively (Fig 18). In case of basolateral to apical side the permeability of irinotecan and SN-38 were $9.09 \pm 0.95 \times 10^{-6}$ cm/sec and $1.91 \pm 0.24 \times 10^{-5}$ cm/sec, respectively. The efflux ratio of irinotecan and SN-38 (figure 18 a) were 15.12 and 4.4, respectively. In both cases, the efflux ratio is higher than 2, which suggests transport of both of these compounds was mediated by efflux transporters according to FDA guidelines.

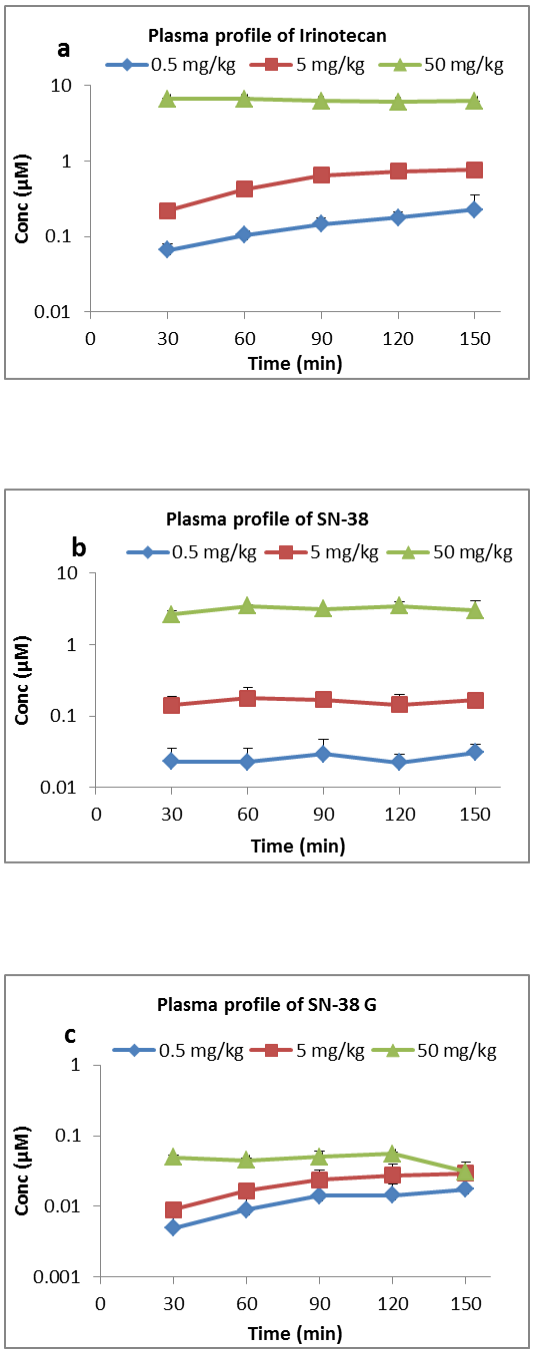


Figure 16: Plasma profile of irinotecan, SN-38 and SN-38 glucuronide at different dose of irinotecan (n=4).

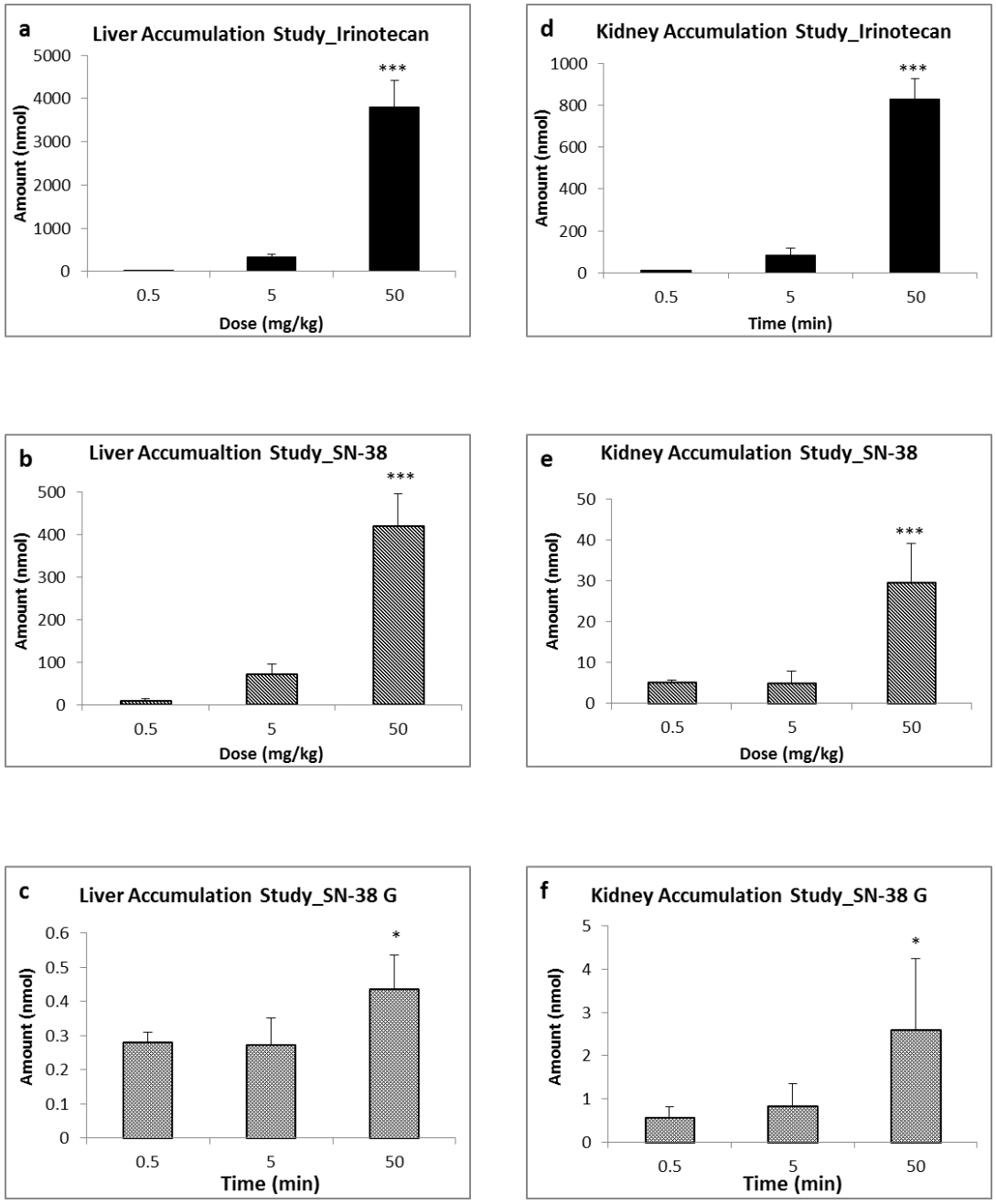
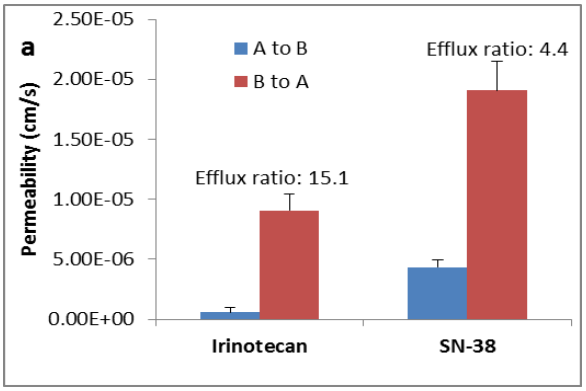
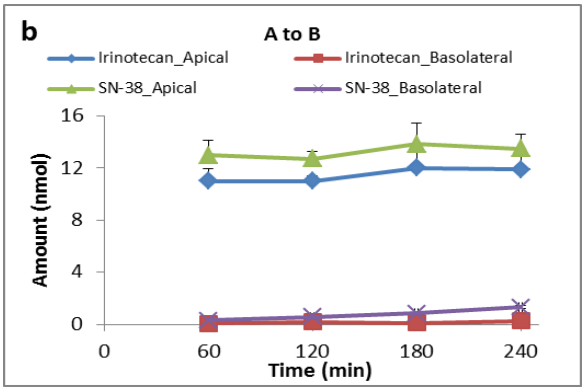


Figure 17: Liver and kidney accumulation study of irinotecan, SN-38 and SN-38 glucuronide at different dose of irinotecan (n=4)





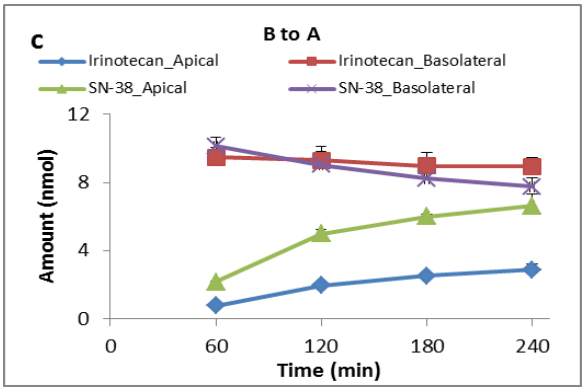


Figure 18: Bidirectional transport of irinotecan (5 µM) and SN-38 (5 µM across Caco-2 cell monolayers (n=3).

4.4.3. Chemical inhibition study of disposition of irinotecan, SN-38 and SN-38 glucuronide using modified *in situ* rat perfusion model:

As Caco-2 cell study indicated that both irinotecan and SN-38 were effluxed by transporters, so we systematically investigated the mechanistic study of disposition of irinotecan using chemical inhibitors of common efflux transporters of different metabolic organs (liver, kidney and intestine) to get a detailed holistic picture of the metabolism, tissue distribution and elimination of irinotecan in different bio-matrices and metabolic organs using the identical modified *in situ* rat perfusion model used in dose response study. For chemical inhibition study, we selected 5 mg/kg intravenous irinotecan which is the equivalent dose of irinotecan causing intestinal toxicity in human. Based on the inhibition constant (K_i) of drug to the particular efflux transporter we have selected cyclosporine A (K_i= 0.46 µM), quercetin (K_i= 8.1 µM), erlotinib (K_i= 0.15 µM) and cimetidine (K_i= 1.1 -2.1 µM) as inhibitors of P- glycoprotein (P-gp), multidrug resistance associated protein (MRP2), breast cancer resistant protein (BCRP) and multidrug and toxic extrusion (MATE) transporters. Depending on the solubility we administered the chemical inhibitors either through intravenous route or perfused a certain segment of jejunum during the entire operation.

4.4.3.1 Biliary excretion rate and cumulative biliary excretion profile of irinotecan:

Figure 19 represented the biliary excretion rate (panel a to c) and cumulative biliary excretion (panel d to f) profile of irinotecan, SN-38 and SN-38 glucuronide after co-administration of chemical inhibitors using *in situ* modified intestinal rat perfusion model. Biliary excretion rate profile suggested that none of the chemical inhibitor were able to

alter the biliary excretion rate of irinotecan (fig 19a) except cyclosporine A which actually increased the excretion of irinotecan at 150 min; whereas in case of SN-38 (fig 19b), cyclosporine A, cimetidine and quercetin significantly decreased the biliary excretion rate at same time point (150 min). In addition, all these three compounds also decreased the biliary excretion rate of SN-38 glucuronide (fig 19c) at multiple time points (90 to 150 min) except erlotinib. Cumulative biliary excretion profile of irinotecan (fig 19d) suggested that both cyclosporine A and quercetin has increased the cumulative biliary excretion of irinotecan significantly, whereas cimetidine has shown mild inhibition. In case of SN-38 (fig 19e), none of the chemical inhibitor was effective to reduce the biliary excretion except cimetidine which reduced approximately 40% biliary excretion of SN-38. After analyzing fig 19f, we found that only cyclosporine A exhibited moderate inhibitory effect (45%) on the biliary excretion of SN-38 glucuronide.

4.4.3.2 Intestinal excretion rate and cumulative intestinal excretion profile of irinotecan:

Contrary to the biliary excretion rate of irinotecan, all chemical inhibitors significantly decreased the intestinal excretion of irinotecan (fig 20a) throughout the entire experiment except for first 30 min. Also in contrast to biliary excretion of SN-38, there were no significant differences of intestinal excretion rate of SN-38 (fig 20b) between the control and treatment group. However, like biliary excretion rate profile, almost all the chemical inhibitors (except quercetin) decreased the intestinal rate of SN-38 glucuronide (fig 20c) at multiple time points. Cumulative intestinal excretion of irinotecan (fig 20d) reflected the same trend like intestinal excretion rate profile where each inhibitor has shown moderate to high inhibition (50-80%). Cyclosporine A also inhibited the

cumulative intestinal excretion of SN-38 and SN-38 glucuronide (fig 20e and 20f) moderately (approximately 40-45%), whereas cimetidine exhibited a mild inhibitory effect (25 to 30%).

4.4.3.3 Urinary excretion rate and cumulative urinary excretion profile of irinotecan:

As we already stated our *in situ* modified rat perfusion model allowed us to monitor urine profile simultaneously alongwith biliary and intestinal profile, we wanted to investigate if chemical treatment have any impact on the urinary excretion of irinotecan and its metabolites during the whole operation. Urinary excretion rate profile of irinotecan (fig 21a) suggested that cyclosporine A and quercetin increased the urinary excretion of irinotecan while cimetidine and erlotinib decreased the excretion significantly. Cumulative urinary excretion plot of irinotecan (fig 21d) also suggested that cyclosporine A significantly increased the urinary excretion (1.6 fold) whereas erlotinib and cimetidine demonstrated strong inhibitory effect (60 to 70%). However, we haven't observed any significant difference of urinary excretion rate as well as cumulative urinary excretion profile of SN-38 (fig 21b and 21e) and SN-38 glucuronide (fig 21c and 21f) after chemical treatment apart from cyclosporine A (3 fold increase). Figure 21c and 21f indicated that none of the chemical has any significant impact on the urinary excretion of SN-38 glucuronide.

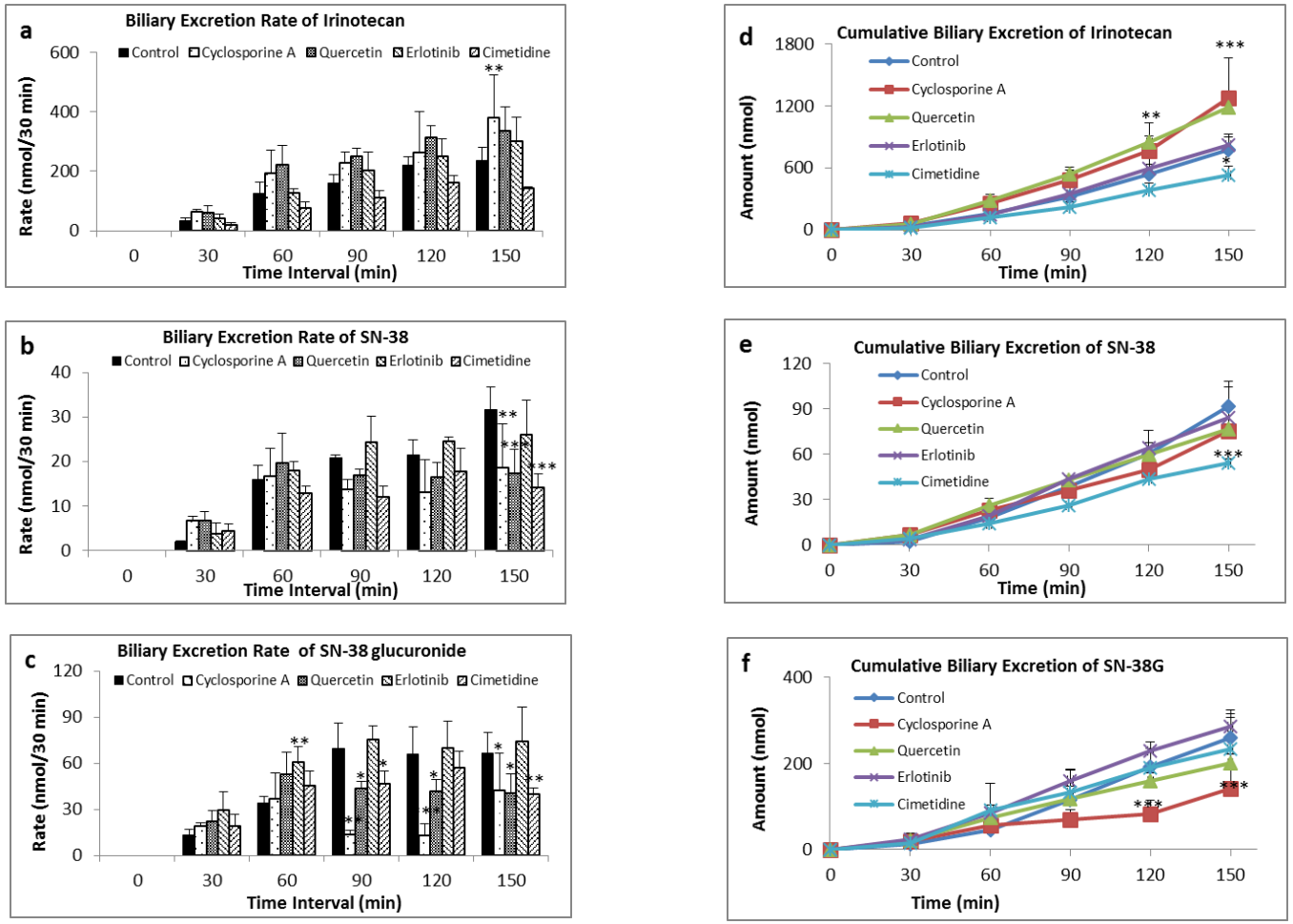


Figure 19: Comparison of the biliary excretion rate and cumulative biliary excretion profiles of irinotecan, SN-38 and SN-38 glucuronide after using chemical inhibitors with the control (n=4).

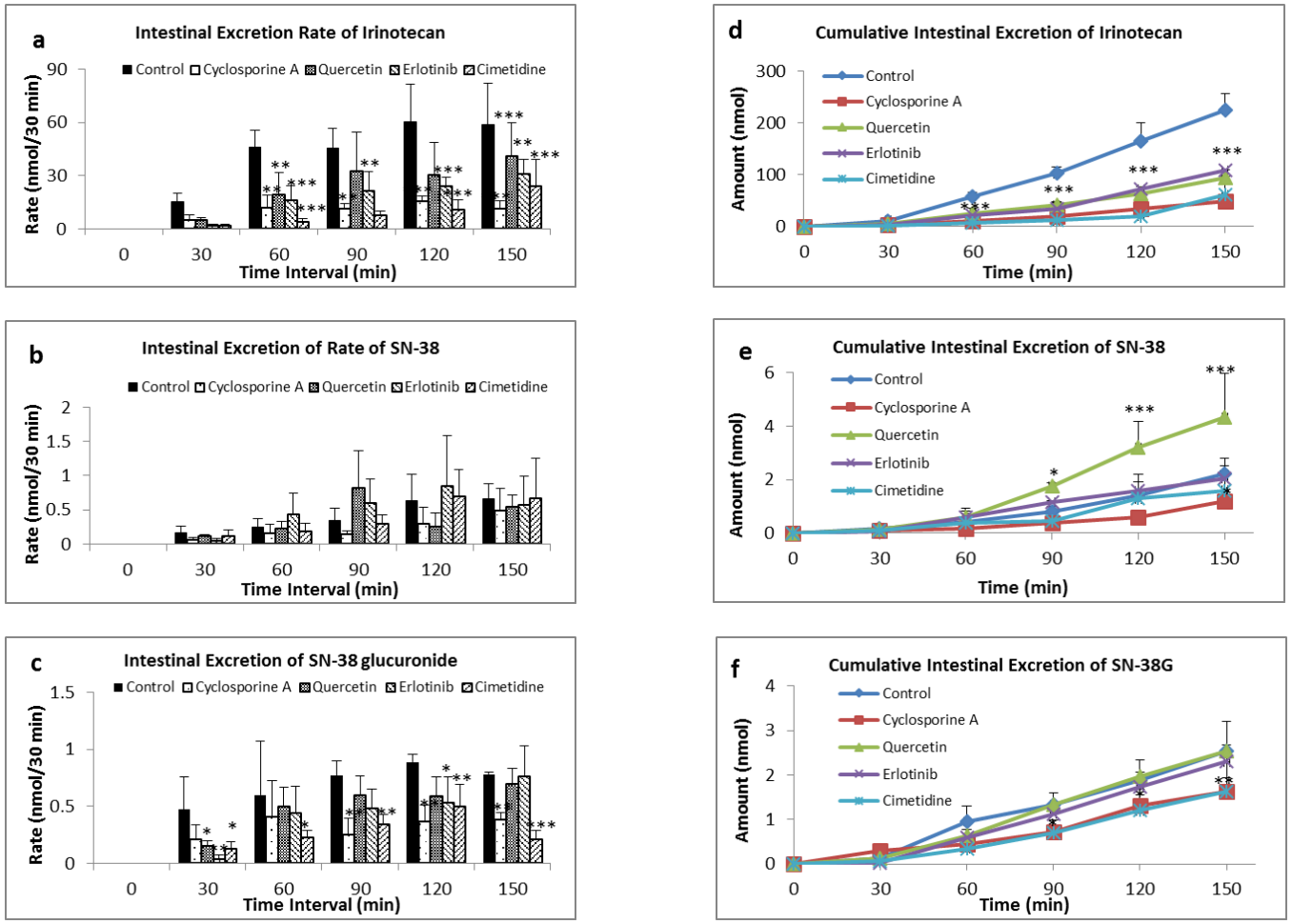


Figure 20: Comparison of the intestinal excretion rate and cumulative intestinal excretion profiles of irinotecan, SN-38 and SN-38 glucuronide after using chemical inhibitors with the control (n=4).

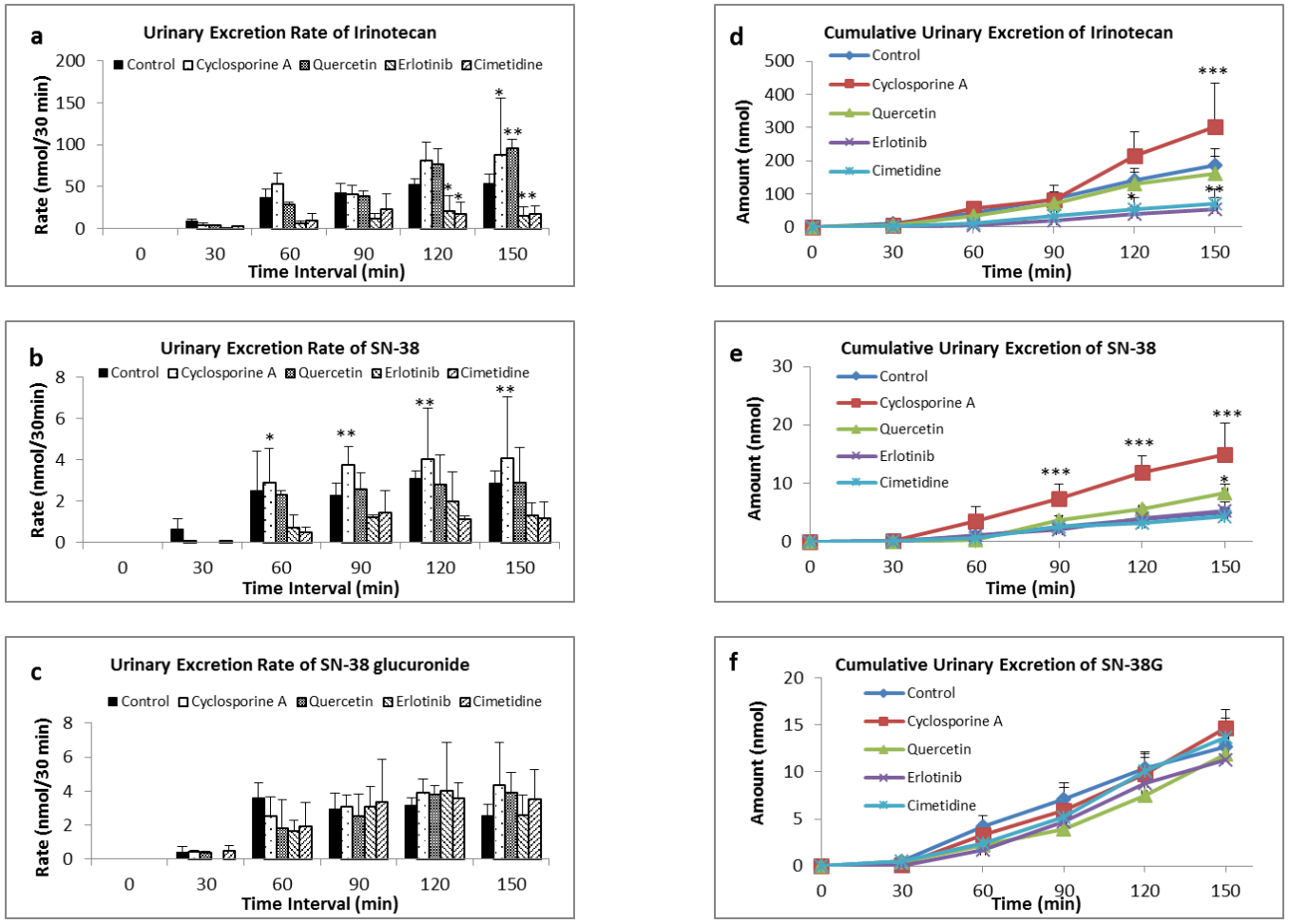


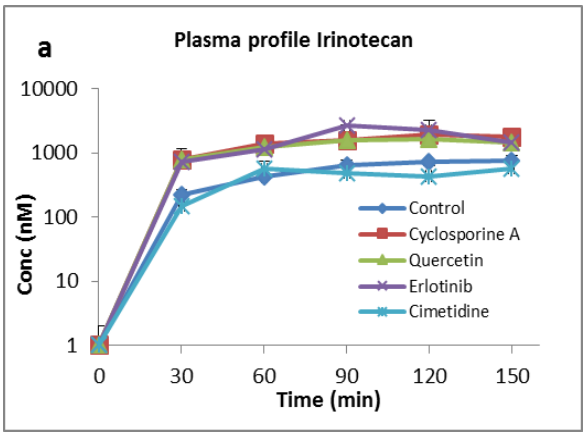
Figure 21: Comparison of the urinary excretion rate and cumulative urinary excretion profiles of irinotecan, SN-38 and SN-38 glucuronide after using chemical inhibitors with the control (n=4).

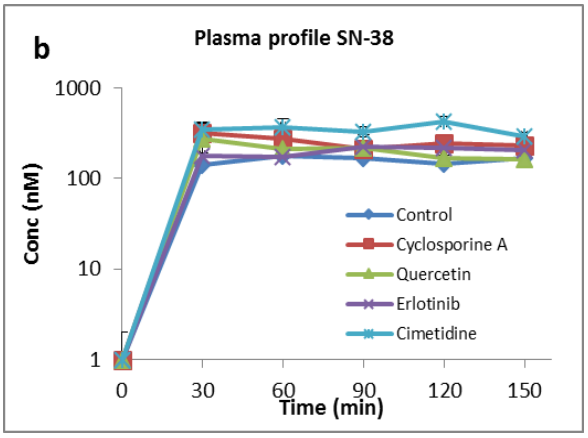
4.4.3.4 Plasma profile of irinotecan:

Plasma profile of irinotecan (fig 22a) described that even after chemical treatment, the plasma concentration of irinotecan and SN-38 reached steady state around 30 min. The chemotherapeutic efficacy of irinotecan remained unaltered after chemical treatment. Table 4 compared the AUC values of irinotecan, SN-38 and SN-38 glucuronide between control and treatment groups in chemical inhibition study.

4.4.3.5 Bio-distribution of irinotecan in liver and kidney:

Bio-distribution study of liver and kidney (fig 23) suggested that none of the chemical inhibitor were able to affect the accumulation of irinotecan, SN-38 and SN-38 glucuronide in liver and kidney. Only in case of SN-38 glucuronide, cimetidine increased the accumulation by 4 fold in liver.





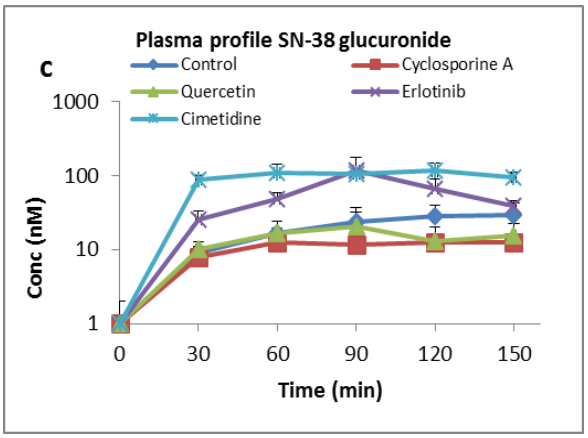


Figure 22: Comparison of the plasma profiles of irinotecan, SN-38 and SN-38 glucuronide after using chemical inhibitors with the control (n=4).

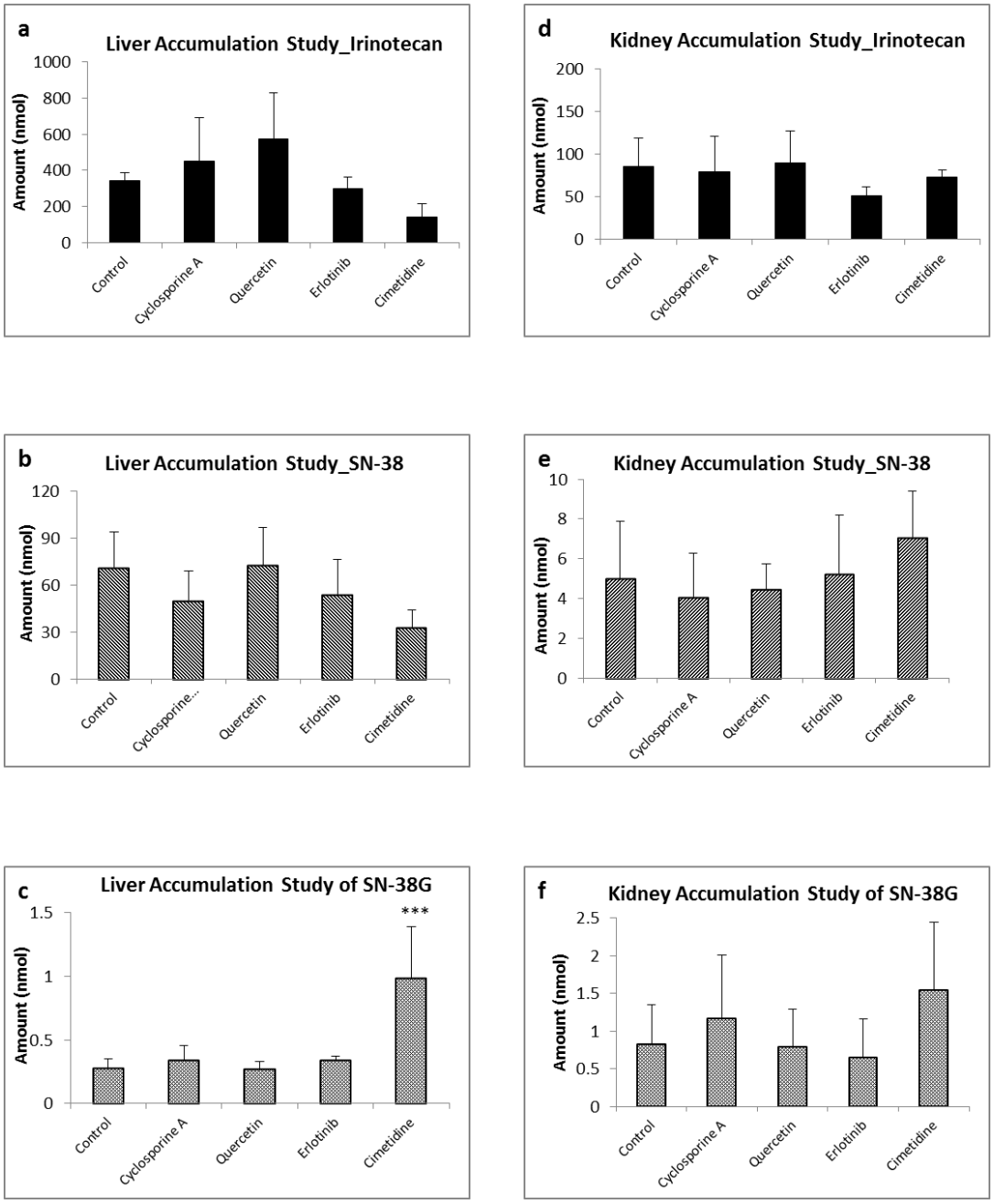


Figure 23: Liver and kidney accumulation study of irinotecan, SN-38 and SN-38 glucuronide at different dose of irinotecan.

4.5. Discussion

Irinotecan exhibits its dose limiting intestinal toxicity as a form of late onset diarrhea which prevents dose intensification in 40% of patient population owing to the higher SN-38 content in intestinal lumen (Hu, Yang et al. 2006; Kurita, Kado et al. 2011). So far, it has been hypothesized that multiple factors such as formation of SN-38 from SN-38 glucuronide by bacterial β-glucuronidase secreted from commensal microbiota, activation of SN-38 from irinotecan by liver and intestinal carboxylesterase enzymes, biliary excretion of SN-38 and SN-38 glucuronide by efflux transporters (P-gp and MRP2) etc. play an important role to elevate the SN-38 content in intestinal lumen which eventually leads to late onset diarrhea (Gupta, Lestingi et al. 1994; Catimel, Chabot et al. 1995; Takasuna, Hagiwara et al. 1996; Ahmed, Vyas et al. 1999; Khanna, Morton et al. 2000). All of these factors implied that both biliary and intestinal excretion of irinotecan and its metabolites contributed significantly in irinotecan induced late onset diarrhea. Apart from biliary and intestinal secretion, determination of urinary excretion is also important as pharmacokinetic studies showed nearly 32% of dose accounted for urinary excretion of irinotecan and its metabolites (Sparreboom, de Jonge et al. 1998; Slatter, Schaaf et al. 2000). In this article, we assessed the pattern of preferential distribution and excretion of irinotecan, SN-38 and SN-38 glucuronide through three major metabolic organs (liver, kidney and intestine) using a modified *in situ* rat perfusion model which will allow us to get a holistic picture of the disposition of irinotecan.

Dose response study demonstrated that at 0.5, 5 and 50 mg/kg dose, approximately 89%, 81% and 74% dose of irinotecan was recovered in the form of irinotecan and its

metabolites in different bio-matrices and metabolizing organs. This study also reestablished the fact is that the major excretion pathway of irinotecan is the biliary route. Figure 24 demonstrated that at different doses, the biliary excretion of irinotecan and its metabolite was approximately 45 – 60% of dose, whereas the urinary and intestinal excretion of irinotecan and its metabolite accounted for 4 to 15% and 3 to 10% of the dose, respectively. We also observed (Table 8) that irinotecan mainly excreted through bile as unchanged form (30 to 45%) rather than its active metabolite SN-38 (1.2 to 5%) and its glucuronide form (2 to 10%). Low biliary excretion of SN-38 and SN-38 glucuronide suggested that the activity of both enzymes CES and UGT towards irinotecan and SN-38 in liver is capacity limited. Liver accumulation study showed that at 5 and 50 mg/kg approximately 14% and 17% of dose of irinotecan was accumulated as unchanged form, whereas 4% of dose was accumulated as unchanged irinotecan in case of 0.5 mg/kg dose. In addition, after calculating the ratio of conc. of irinotecan in liver and plasma at 150 min, we found that the ratio increased steadily from 2.06 (at 0.5 mg/kg) to 4.04 (50 mg/kg dose). Based on these observations, we are proposing atheory that once biliary secretion of irinotecan gets saturated, liver will act as a ‘storage organ’ or ‘depot’ of irinotecan. This theory may explain the reason of liver toxicity such as steatohepatitis in 5-20% of patient population who are undergoing irinotecan chemotherapy (Cleary, Tanabe et al. 2009).

In addition, our dose response study also emphasized that the excretion of irinotecan, SN-38 and SN-38 glucuronide in liver at different doses is efflux transporter mediated. After calculating the ratio of the concentration of irinotecan in bile compared to plasma at

steady state we found that the value ranges from 450 to 800, which clearly indicated the high efficiency of efflux transporters. Similalry, in case of SN-38 and SN-38 glucuronide the bile to plasma ratio ranges from 35 to 350 fold and 300-5000 fold at different doses indicating the influence of efflux transporters on the transport of SN-38 and SN-38 glucuronide. We observed the similar trend in urine for irinotecan and SN-38 glucuronide at different doses. However, in case of intestinal perfusate of irinotecan and SN-38 glucuronide, apart from 50 mg/kg dose the ratio of intestinal perfusate concentration over plasma concentration was 1 signifies the mechanism is to be passive absorption.

Our modified *in situ* rat perfusion model has certain advantages over the classical perfusion model. Firstly, we can utilize our model to determine the relative contribution of different metabolic organs on the excretion profile of any drug candidate/xenobiotic performing one single experiment, whereas for other models several experiments needed to be conducted which undoubtedly increases the chance of getting variable results. Secondly, this model enables us to compare how the pattern of excretion and distribution of a drug and its metabolites with respect to its plasma concentration can change over different doses. For an example, in this study the ratio of SN-38 glucuronide in liver with respect to plasma remains steady (0.08 to 0.11) at different doses; whereas in case of kidney the ratio increased by 2 fold at 50 mg/kg dose. Similarly, after calculating the ratio of irinotecan in urine and perfusate with respect to plasma we found out that in case of urine the ratio has increased at 5 mg/kg by 3 fold, whereas the ratio stayed same for intestinal route (1.3). However, upon increment of dose to 50 mg/kg, the ratio for intestinal route increased to approximately 5 fold, whereas the ratio remained

constant for urine implying the saturation of urinary excretion route as well as elevation of intestinal excretion of irinotecan with the increment of dose at the same time. All of these ratios were calculated based on the concentration measured at 120-150 min, where already the steady state have been achieved which gives us a realistic picture of the *in vivo* condition.

In addition, our modified *in situ* rat perfusion model also allows us to devise different strategies to modify the excretion pathways of irinotecan and its metabolites by modulating different efflux transporters. As Caco-2 cell study confirmed that efflux transporters are playing an active role to transport both irinotecan and SN-38, so we systematically investigated the impact of efflux transporters on the disposition of irinotecan using our developed *in situ* rat perfusion model by co-administering chemical inhibitors of efflux transporters with irinotecan. We selected 5 mg/kg dose of irinotecan as control dose in this chemical inhibition study as it is more close to the human equivalent dose. As the chemical inhibitors have broad substrate specificity, so for the sake of understanding we assumed that any inhibitor shows greater than 70% inhibition will be called as strong inhibitor in this study. Similarly, inhibitors showing 30% to 70% inhibition will be called as moderate inhibitor, whereas less than 30% of inhibition will be termed as mild effect. According to this, we found that three chemicals cyclosporine A (18%), quercetin (16%) and cimetidine (41%) exhibited mild to moderate inhibition of cumulative biliary excretion of SN-38. In addition, both quercetin (23%) and cyclosporine A (45%) also inhibited cumulative biliary excretion of SN-38 glucuronide moderately. On the contrary, they increased the cumulative biliary excretion of irinotecan (50-65%)

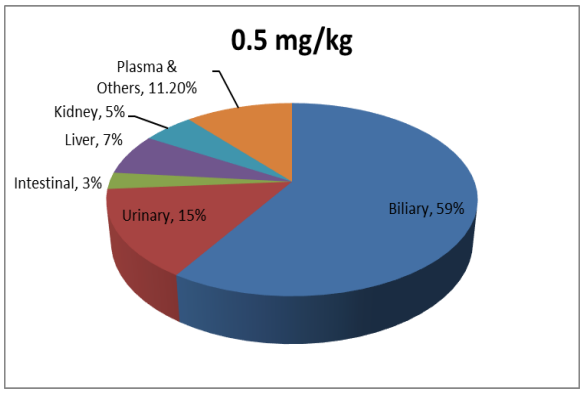
whereas cimetidine showed mild inhibition (32%) which suggested that apart from P-gp and MRP2 transporters, irinotecan might be effluxed by MATE-1 transporter. Similarly, cumulative urinary excretion profile of irinotecan also supported the fact as cimetidine exhibited quite strong inhibition of urinary excretion of irinotecan (63%). Based on the previous cellular experiment and computational modeling result, it has been demonstrated that irinotecan acted as both substrate and inhibitor of MATE-1 receptor in liver and kidney (Grottker, Rosenberger et al. 2011; Wittwer, Zur et al. 2013). Our *in situ* modified rat perfusion model for the first time demonstrated the fact that MATE-1 transporter also played an important role in the disposition of irinotecan, especially in liver and kidney. However, further investigations are warranted to determine how MATE-1 can influence the pharmacokinetics of irinotecan.

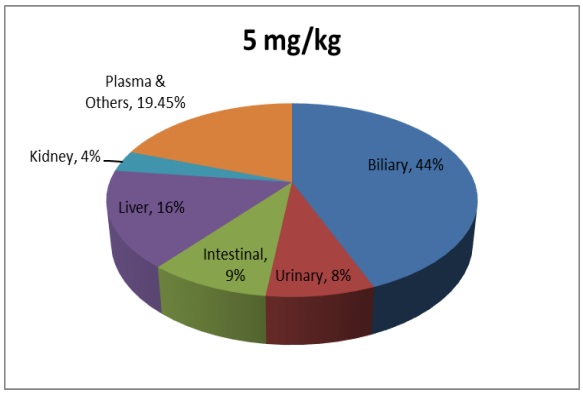
As the therapeutic index of irinotecan is quite narrow, so monitoring the plasma content level of SN-38 is quite necessary. High plasma level of SN-38 may exhibit neutropenia and myelosuppression, whereas low SN-38 concentration in plasma signifies loss of efficacy (Rothenberg 1998; Kim and Innocenti 2007). In this regard, our *in situ* modified rat perfusion model has the provision of monitoring the plasma content of test drug throughout the operation at specific time interval. Dose response study (table 3) indicated that the AUC of irinotecan, SN-38 and SN-38 glucuronide increased linearly with the increment of dose. Lastly, chemical inhibition study (table 4) indicated that AUC of irinotecan in plasma was significantly increased by all of the chemicals by 2 to 2.5 fold except cimetidine. On the other hand, only cimetidine among all the chemicals elevated the AUC value of SN-38 by 2.5 fold which might be speculated as the induction of

neutropenia. However, according to the study conducted by Hirose et al. low plasma concentration of SN-38 glucuronide with respect to SN-38 is the indication of low UGT1A1 activity which may lead to neutropenia due to low plasma SN-38 clearance (Hirose, Kozu et al. 2012). However, in our study as cimetidine also increased the AUC of SN-38 glucuronide by 4 fold, so the net ratio of SN-38 glucuronide/SN-38 (0.29 vs 0.18) was similar after the cimetidine treatment which indicated the UGT activity remained constant.

Studies indicated that both cyclosporine A and quercetin have been tried before to explore the role of efflux transporters on the disposition of irinotecan. Though contribution of cyclosporine on the biliary and intestinal excretion of irinotecan has been investigated by using an *in situ* rat perfusion model, the quantification of SN-38 glucuronide wasn't reported in any of the bio-matrices. From the dose response study, we have observed that the contribution of SN-38 glucuronide to the total dose is as important (2-11%) as SN-38 (3-11%), so determination of SN-38 glucuronide is really worthwhile to get a holistic picture of the disposition of irinotecan. Moreover, as cyclosporine is a well-known nephrotoxic drug, so determination of the urinary excretion profile of irinotecan and its metabolites is also imperative before clinical use. As our model *in situ* rat perfusion model has the provision of determining the urinary profile of test substance so it might be utilized as a screening tool to characterize a drug before clinical trial. In case of quercetin, only biliary excretion profile of irinotecan and SN-38 has been explored to whereas in this study we determined biliary, urinary and intestinal excretion profile of irinotecan, SN-38 as well as SN-38 glucuronide.

In conclusion, our modified *in situ* rat perfusion model demonstrates a holistic picture (mass balance) of disposition of anticancer drug irinotecan and its metabolites (SN-38 and SN-38 glucuronide) by determining the contribution of different metabolic organs (liver, kidney and intestine) as well as allows us to observe/compare how irinotecan and its metabolites is preferentially eliminated through different excretion pathways (biliary, intestinal and urinary) at different doses. In addition, chemical inhibition studies indicate that this particular model can be applied to drug-drug interaction studies (DDI), where we can determine how the disposition profile of a drug (victim) may be significantly altered by another drug (perpetrator). Apart from DDI studies, we can also elucidate the contribution of a particular excretion pathway on the disposition of a drug candidate/xenobiotic as this particular model has the ability to quantify the extent of excretion of a compound and its metabolite through different excretion pathways simultaneously.





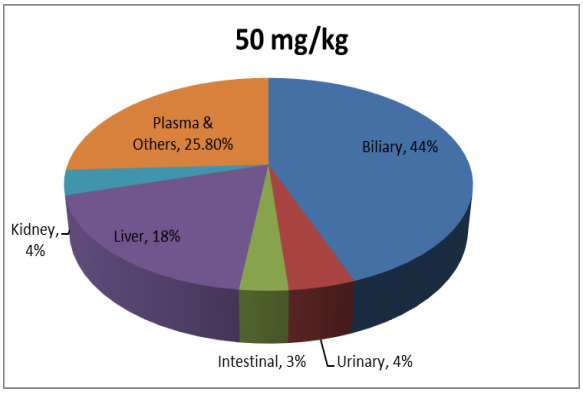
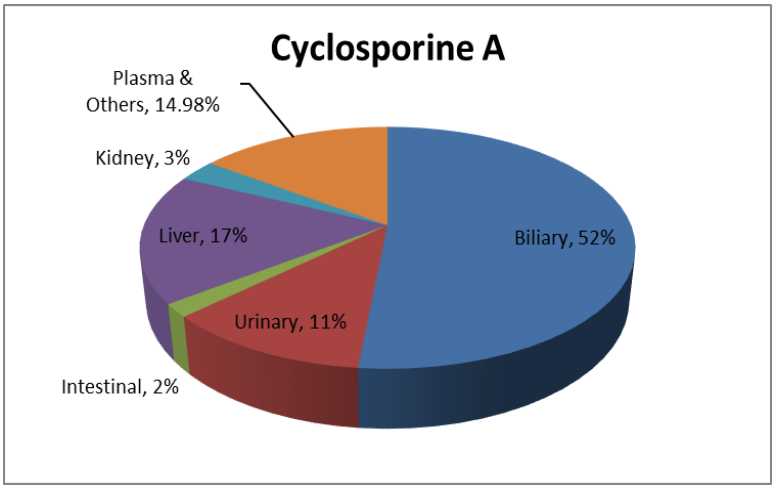
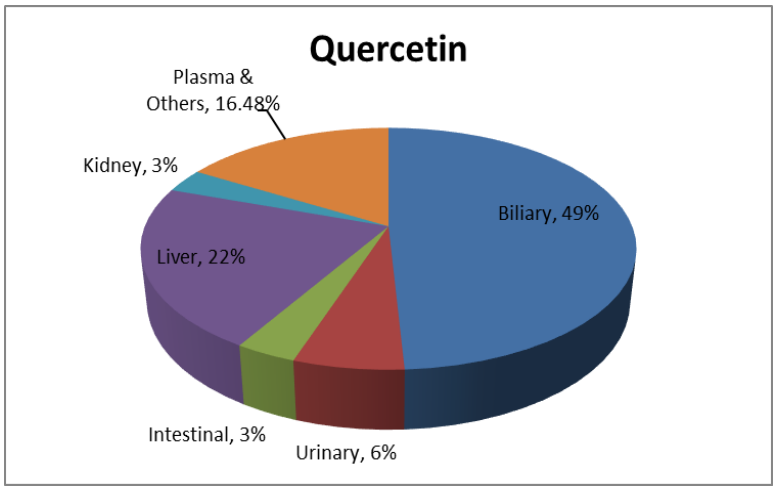
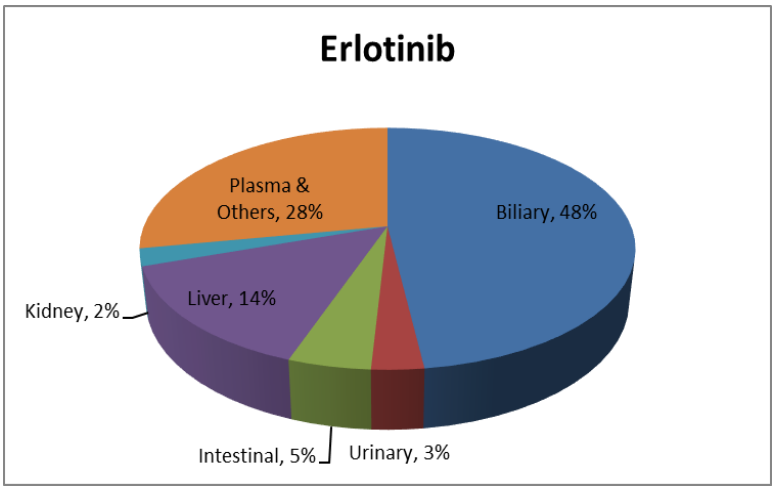


Figure 24: Mass balance study of irinotecan, SN-38 and SN-38 glucuronide at different dose of irinotecan.







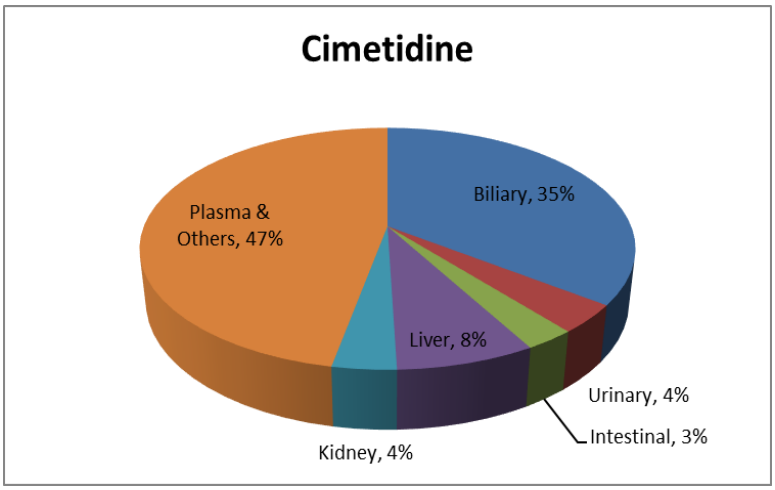


Figure 25: Mass balance study of irinotecan, SN-38 and SN-38 glucuronide after using different chemical inhibitors.

Table 8: Percentage excretion based on dose of irinotecan, SN-38 and SN-38 glucuronide in Bile, Perfusate, Urine, Liver and Kidney after dose response study

Routes of elimination	0.5 mg/kg			5 mg/kg			50 mg/kg		
	Irinotecan	SN-38	SN-38G	Irinotecan	SN-38	SN-38G	Irinotecan	SN-38	SN-38G
Bile (%)	45.98	4.75	8.25	30.24	3.57	10.12	41.06	1.19	2
Perfusate (%)	2.36	0.24	0.21	8.74	0.09	0.1	3.19	0.03	0.02
Urine (%)	12.50	1.34	0.96	7.27	0.19	0.49	4.25	0.06	0.12
Liver accumulation (%)	3.87	2.99	0.1	13.38	2.78	0.01	16.65	1.84	0.002
Kidney accumulation (%)	3.31	1.76	0.19	3.33	0.19	0.03	3.65	0.13	0.01

Table 9: Percentage excretion of irinotecan, SN-38 and SN-38 glucuronide in Bile, Perfusate, Urine, Liver and Kidney after chemical inhibition study

Routes of elimination	Control			Cyclosporine A			Quercetin			Erlotinib			Cimetidine		
	Irinotecan	SN-38	SN-38G	Irinotecan	SN-38	SN-38G	Irinotecan	SN-38	SN-38G	Irinotecan	SN-38	SN-38G	Irinotecan	SN-38	SN-38G
Bile (%)	30.24	3.57	10.12	44.03	2.6	4.94	39.83	2.58	6.72	33.2	3.4	11.56	22.87	2.34	10.07
Perfusate (%)	8.74	0.09	0.1	1.65	0.04	0.06	3.15	0.14	0.09	4.39	0.08	0.09	2.65	0.07	0.07
Urine (%)	7.27	0.19	0.49	10.41	0.51	0.51	5.4	0.28	0.40	2.2	0.21	0.46	3.01	0.18	0.59
Liver (%)	13.38	2.78	0.01	15.59	1.73	0.01	19.27	2.44	0.01	12.17	2.19	0.01	6.2	1.42	0.04
Kidney (%)	3.33	0.19	0.03	2.75	0.14	0.04	3.02	0.15	0.03	2.08	0.21	0.03	3.15	0.30	0.07

Table 10: AUC (±SD) (μM· min) of irinotecan, SN-38 and SN-38 glucuronide in plasma at 150 min after dose response study

0.5 mg/kg			5 mg/kg			50 mg/kg		
Irinotecan	**SN-38**	**SN-38G**	**Irinotecan**	**SN-38**	**SN-38G**	**Irinotecan**	**SN-38**	**SN-38G**
6.11 (2.52)	0.79 (0.25)	0.48 (0.24)	22.46 (2.46)	4.68 (1.2)	0.86 (0.37)	185.85 (2.33)	97.13 (25.14)	1.29 (0.11)

Table 11: AUC (±SD) (μM· min) of irinotecan, SN-38 and SN-38 glucuronide in plasma at 150 min after chemical inhbition study

Control			Cyclosporine A			Quercetin			Erlotinib			Cimetidine		
Irinotecan	**SN-38**	**SN-38G**	**Irinotecan**	**SN-38**	**SN-38G**	**Irinotecan**	**SN-38**	**SN-38G**	**Irinotecan**	**SN-38**	**SN-38G**	**Irinotecan**	**SN-38**	**SN-38G**
22.46 (2.46)	4.68 (1.2)	0.86 (0.37)	55.54 (7.42)	7.08 (0.37)	0.38 (0.11)	46.49 (5.42)	5.03 (0.17)	0.43 (0.43)	55.66 (16.25)	6.4 (1.41)	1.56 (0.89)	15.14 (6.76)	10.73 (1.46)	3.16 (1.39)

Chapter 5 Modulation of disposition of irinotecan and its metabolites using selected chemicals and natural products

5.1. Abstract

Irinotecan is used as a single agent or in combination with other drugs to treat metastatic colorectal cancer. However its usage is largely limited as it exhibits late onset diarrhea due to the high accumulation of SN-38 in colon. The objective of this study was to demonstrate if selected chemical inhibitor (cimetidine) and traditional Chinese herbal formulation (Xiaochaihutang and PHY 906) can decrease the colonic content of SN-38 by decreasing biliary and intestinal excretion of SN-38 resulting in alleviation of irinotecan induced intestinal toxicity. For this purpose, at first we utilized a modified *in situ* rat perfusion model to observe if cimetidine, Xiaochaihutang (XCHT) and PHY906 can significantly alter the disposition of irinotecan and its metabolites. Our modified *in situ* rat perfusion model indicated that both cimetidine and XCHT can decrease the biliary and intestinal excretion of SN-38 and SN-38 glucuronide by 28-90%. Secondly, we performed a rat pharmacokinetic study to investigate if coadministration of cimetidine and XCHT with irinotecan can reduce the fecal content of SN-38 without altering the efficacy of the chemotherapy. Results showed that both XCHT and cimetidine can decrease the fecal content of SN-38 by 40-50%. To the best of our knowledge, this is the first time cimetidine and Chinese herbal formulation XCHT have displayed their ability to decrease both biliary and fecal excretion of SN-38 which might lead to amelioration of irinotecan induced late onset diarrhea. However, further investigations are warranted to prove their effectiveness in irinotecan toxicity using a preclinical diarrhea model.

5.2. Introduction

Irinotecan (CPT-11), a semi-synthetic derivative of camptothecin, is regarded as one of the firstline therapy against metastatic colorectal cancer in combination with other agents such as 5-fluorouracil, leucovorin etc. (Saltz, Cox et al. 2000; Mathijssen, van Alphen et al. 2001). Apart from colorectal cancer, it has also demonstrated its clinical efficacy in malignancies of lung, pancreatic, cervical, ovarian, etc. (Ohno, Okada et al. 1990; Fukuoka, Niitani et al. 1992; Rosen 1998). Irinotecan, through the action of liver and intestinal carboxylesterase enzymes, generates its active metabolite SN-38 which inhibits the activity of mammalian DNA topoisomerase I by forming a complex during DNA replication leading to cell death (Stewart, Leggas et al. 2004). Bio-distribution studies of irinotecan indicated that liver is the primary important organ which influence predominantly the disposition of irinotecan and its active metabolite SN-38 (Iyer, Ramirez et al. 2002; Luo, Paranjpe et al. 2002; Itoh, Itagaki et al. 2005). Mechanistic studies demonstrated that elevated content of SN-38 in intestinal lumen owing to the excessive biliary and intestinal excretion of SN-38 and its phase II metabolite SN-38 glucuronide is considered to be one of the major factors responsible for irinotecan induced intestinal toxicity - late onset diarrhea which limits usage of irinotecan in at least 10 to 20% of patient population (Mathijssen, van Alphen et al. 2001; Yang, Hu et al. 2005). Though different drugs such as loperamide, octreotide etc. are being used to treat irinotecan induced late onset diarrhea in clinical settings, none of these can completely reduce the severity of the diarrhea (Ruppin 1987; Benson, Ajani et al. 2004; Rosenoff 2004).

Various experimental studies provided the direct evidence that transportation of irinotecan, SN-38 and SN-38 glucuronide from systemic circulation to the bile and intestine was mediated by predominant efflux transporters (P-gp and MRP2) in combination (Chu, Kato et al. 1997; Sugiyama, Kato et al. 1998; Smith, Figg et al. 2006). Mechanistic studies hypothesized that multiple factors such as higher biliary excretion of SN-38 and SN-38 glucuronide mediated through different efflux transporters, deglucuronidation of inactive SN-38 glucuronide to toxic SN-38 mediated by bacterial β-glucuronidase secreted from commensal microbiota, conversion of prodrug irinotecan to the active metabolite SN-38 in gut by intestinal carboxylesterase enzymes (CES) and enterohepatic recycling of irinotecan and SN-38 causes elevation of colonic content of SN-38 which directly damages the intestinal epithelium causing late onset diarrhea (Gupta, Lestingi et al. 1994; Catimel, Chabot et al. 1995; Takasuna, Hagiwara et al. 1996; Ahmed, Vyas et al. 1999; Khanna, Morton et al. 2000; Ma and McLeod 2003). Owing to the direct effect of efflux transporters on the occurrence of late onset diarrhea, several approaches have already been taken to reduce the severity of the late onset of diarrhea by strategically modulating the function of the enzymes and efflux transporters (Chester, Joel et al. 2003; Innocenti, Undevia et al. 2004; Desai, Kindler et al. 2005; Vasudev, Jagdev et al. 2005). For an example, cyclosporine A and probenecid have been used in preclinical studies to ameliorate irinotecan associated intestinal toxicity by inhibiting the activity of P-gp and MRP2 transporters (Mathijssen, van Alphen et al. 2001; Horikawa, Kato et al. 2002).

Since ancient times, herbal medicines have been used extensively for the treatment of cancer. Owing to its safety and lack of adverse effect, herbal medicines specially traditional Chinese medicines (TCM) continue to be widely used in world under the division of complementary and alternative medicine (CAM) (Eisenberg, Davis et al. 1998). Generally the activity of Traditional Chinese medicine (TCM) is solely depends on the interaction of multiple components of the TCM combination acting in a synergistic manner to enhance oral absorption, improve clinical efficacy, and reduce toxicity. Herbal medicines are broadly utilized in cancer chemotherapy as anticancer agents, adjuvant to prevent and/or reduce toxicity from anticancer agents, and as chemoprevention agents to reduce the risk of development and progression of cancer (Wong, Sagar et al. 2001). For an example, earlier reports described that kampo medicines were utilized along with irinotecan-based chemotherapy in clinical trial to ameliorate irinotecan induced late onset diarrhea (Katoh, Yoshioka et al. 2009; Chikakiyo, Shimada et al. 2012).

PHY906 is a novel Chinese herbal preparation which exhibits various pharmacological activities like antiviral, immunologic, analgesic, vasodilatory, hepatoprotective, antioxidant, and appetite stimulatory effects (Kummar, Copur et al. 2011). This particular preparation is composed of 4 main herbs, *Scutelleria baicalensis Georgi, Paeonia lactiflora Pall.,Glycyrrhiza uralensis Fisch.,* and *Ziziphus jujuba Mill*. PHY 906 was used extensively treat a variety of gastrointestinal side effects such as diarrhea, abdominal cramps, vomiting, as well as fever and headache (Kummar, Copur et al. 2011). Owing to its cryoprotective effect and lack of severe side effects, it has also been used in preclinical and clinical studies to investigate whether it can reverse irinotecan induced

late onset diarrhea. It was found to decrease the incidence of late onset diarrhea as well as potentiated the anticancer activity of irinotecan by promoting the expression of intestinal stem cells (Lam, Bussom et al. 2010; Kummar, Copur et al. 2011). However, in order to receive the marketing approval from regulatory authorities an appropriate phase II and III study is required. Like PHY 906, Chinese medicine Xiaochaihutang (XCHT, known as Sho-Saiko-To or SST or TJ-14 in Japan) is also undergoing clinical trial to get the approval from regulatory authorities for the treatment of chronic hepatitis. It is mainly composed of seven herbs namely, *Radix Bupleuri*, *Radix Scutellariae*, *Rhizoma Pinelliae*, *Radix Ginseng*, *Radix Glycyrrhizae*, *Rhizoma Zingiberis Recens*, and *Fructus Jujubae*. Preclinical studies indicated that XCHT and its main chemical components such as baicalin, baicalein, glycyrrhizin, and saikosaponin-D demonstrated antiproliferative activity against hepatocellular carcinoma, decreased hepatic fibrosis and regenerated liver expression in animal models (Dai, Yang et al. 2008; Song, Kim et al. 2014; Takahashi, Soejima et al. 2014). The safety profile of XCHT is already well characterized as it is already available in market.

The main objective of our study is to determine if our selected chemical and herbal formulations have the ability to decrease the colonic content of SN-38. To test this hypothesis, we utilized the modified *in situ* rat perfusion model to observe if these agents can decrease the biliary and intestinal excretion of SN-38 and SN-38 glucuronide. Furthermore, we also performed the *in vivo* pharmacokinetic study to determine if the fecal excretion of SN-38 decreased after treating with selected inhibitors which showed efficacy in our *in situ* modified rat perfusion model. We utilized XCHT and PHY 906 in

our modified *in situ* rat perfusion model as one of the main component of these two herbs is baicalin which is a β-glucuronidase inhibitor (Takasuna, Kasai et al. 1995). Lastly, we also used cimetidine as chemical inhibitor in the pharmacokinetic study as the inhibitory effect of this drug to the biliary and intestinal excretion of SN-38 and SN-38 glucuronide was better compared to other chemical inhibitors we used in our *in situ* modified rat perfusion model (chapter 5).

5.3. Materials and methods

5.3.1. Materials

Irinotecan, SN-38, cimetidine, camptothecin (CPT), uridine-5'-diphosphate-β,D-glucuronic acid ester (UDPGA), D-saccharic-1,4-lactone monohydrate, magnesium chloride, hanks' balanced salt solution (powder form) and formic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Expressed human UGT isoforms (UGT1A1) was purchased from BD Biosciences (Woburn, MA, USA). Solid phase extraction (C18) columns were purchased from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile, methanol and water (LC-MS grade) were purchased from EMD (Gibbstown, NJ, USA). Water was deionized by a Milli-Q water purification system of Millipore (Bedford, MA, USA). Intravenous irinotecan hydrochloride injection (20 mg/ml) was purchased from Teva Pharmaceuticals (Pearl River, NY, USA). All other materials (typically analytical grade or better) were used without further purification.

5.3.2. Biosynthesis of SN-38 glucuronide

SN-38 glucuronide was biosynthesized using human expressed UGTs (UGT1A1 isoform). In this study, SN-38 was incubated with UGT1A1 for 24 hours which resulted in the formation of 95.8% SN-38 glucuronide. We separated the residual aglycone (SN-38) from the mixture after employing liquid-liquid extraction utilizing dichloromethane (DCM). Then each 5 ml of processed samples were applied to a C18 solid phase extraction column. After washing out the salt, 1 ml of methanol was used to elute the SN-38 glucuronide. The eluted fractions of methanol were collected and air dried, and the residue was reconstituted with 100 µl of 50% methanol-water to concentrate SN-38 glucuronide. In each step, we measured the respective peak area of SN-38 and SN-38 glucuronide in UPLC to calculate the purity of SN-38 glucuronide in the mixture. Apart from UPLC quantification, we also utilized MS/MS spectrum of SN-38 glucuronide to confirm its presence in the solution.

5.3.3. Animal

Male Wistar rats (6-10 weeks, Body weight between 250 to 280g, n = 6) were purchased from Harlan Laboratory (Indianapolis, IN). Rats were kept in an environmentally controlled room (temperature: 25 ± 2°C, humidity: 50 ± 5%, 12 h dark-light cycle) for at least 1 week before the experiments. The rats were fasted overnight before the day of the experiment.

5.3.4. Animal surgery

The procedures were approved by the University of Houston's Institutional Animal Care and Uses Committee (IACUC). Before starting the surgery, at first isoflurane-oxygen

mixture was used to anaesthetize wistar rats and then 50% dose of urethane (2g/kg) was injected through intramuscular route. After injecting urethane, we waited for 30-45 minutes to inject the rest of the urethane to make sure that the rat is not showing any kind of unusual behavior. We started to perform our surgery once we are certain that the rat is totally unconscious. At first, the rat was put on a heating blanket under a heating lamp to stabilize its normal body temperature. The skin of neck and abdomen was properly shaved for surgery and cannula insertion. For bile duct cannulation, a 5 cm abdominal midline incision is made to locate duodenum and then the fatty tissues are removed from the surrounding tissues to separate the bile duct from the surface. Next, we inserted a tubing (PE10) after making a small cut with a micro vascular scissor and secured the tubing with a surgical suture. To cannulate one segment of jejunum, we inserted an inlet cannula at the beginning of the jejunum whereas the outlet cannula was inserted about 20-25 cm below the inlet one. After suturing both of the cannulas, we put the intestine into the abdominal cavity which was covered by a saline-wetted cotton towel to prevent excessive bleeding and/or moisture loss. In addition, the inlet cannula was insulated and kept warm at 37°C by a circulating water bath to keep the temperature of the perfusate constant. For urinary bladder cannulation, first we located the top portion of the urinary bladder and make a small cut with a micro vascular scissor. Then we inserted a soft tubing (PE10) through the cut and secured the tubing with a surgical suture which will allow urine to flow into the collection vials. Lastly, for cannulation of jugular vein we made a 1.5 cm incision on the left neck. Then we removed the fatty tissue from the jugular vein surface after dissecting different layers of tissues that cover the vein (e.g., superficial fascia and shallow muscularis). After locating and

identifying the jugular vein, we inserted a tubing (PE20) through a small incision made by a microvascular scissor to collect blood from jugular vein.

5.3.5. Intestinal perfusion experiment

This modified *in situ* rat perfusion experiment is a single-pass perfusion method. One segment of jejunum was perfused with a HBSS buffer using an infusion pump (model PHD 2000; Harvard Apparatus Inc., Holliston, MA) at a flow rate of 0.191 ml/min for 150 min. After a 30-min washout period, which was usually sufficient to achieve the steady-state absorption, four samples were collected from the outlet cannula periodically (every 30 min). Apart from perfusate samples, we also collected bile (0.4 ml), urine (0.2 ml), and blood samples (0.1 ml) at 30 minute interval. Blood samples were centrifuged at 5000 rpm for 5 min and the pellet was discarded. The outlet concentrations of irinotecan, SN-38 and SN-38 glucuronide in bile, intestinal perfusate and plasma were determined by UPLC-MS/MS; whereas all urine samples containing irinotecan and its metabolites were determined by UPLC. In this study, we infused cimetidine (5 mg/kg iv) through jugular vein alongwith 5 mg/kg irinotecan, whereas XCHT (100 μM baicalin) and PHY 906 (100 μM baicalin) perfused through the segment of jejunum after dissolving in HBSS buffer.

5.3.6. Tissue homogenization

Liver and kidney were excised from the sacrificed rats and stored frozen at −80°C in polypropylene tubes until homogenization. The frozen tissues were thawed, chopped and weighed at 4°C. Accurately 50 mg of chopped tissue was homogenized in 2 ml of ice-cold homogenizing solution (pH 7.4) containing 10 mM potassium phosphate, 250

mM sucrose and 1 mM EDTA dehydrate with a polytron tissue homogenizer. Homogenization was paused 20 s after every 30 s of homogenization at a medium speed. The homogenization was repeated 3 – 4 times until a uniform homogenate was obtained. Final tissue extract was stored at approximately −80°C prior to analysis. The homogenizer probe was washed sequentially with water, methanol and water after every homogenization.

5.3.7. *In vivo* rat pharmacokinetic study design

Irinotecan was administered at a dose of 5 mg/kg via intravenous injection through the tail vein. Blood samples (about 20 -50 µL) were collected in heparinized tubes at 0, 15, 30, 60, 120, 240, 360, 480, and 1440 min post-injection, via tail snip with isoflurane as anesthetic. Urine samples were collected at 4, 8 and 24 hours; whereas feces were collected at 24 hour. Plasma samples were prepared and stored at - 80°C until analysis. The procedures were approved by the University of Houston's Institutional Animal Care and Uses Committee (IACUC).

5.3.8. Sample preparation

We diluted bile samples (5 µl) 100 times by 50% aqueous methanol solution and added 50 µl 0.1 µM CPT in 50% methanol-water as internal standard to the solution. In case of urine and perfusate samples, we diluted 10 and 50 µl samples with 100 and 50 µl 50% methanol-water, respectively and added 50 µl of testosterone in 94% acetonitrile - 6% acetic acid mixture (internal standard) and 100 µl 0.1 µM CPT in 50% methanol-water (internal standard). After addition of 50% methanol-water solution and internal standard, all the processed samples (bile, urine and perfusate) were vortexed, centrifuged for 15

min at 15500 rpm and 10 µl supernatant was injected to UPLC-MS/MS and UPLC for analysis. Plasma, liver and kidney homogenate samples (20 µl) were spiked with 20 µl of internal standard (CPT in 50% methanol, 100 nM) and vortexed for 1 min after extracted with 50% methanol-acetonitrile solution in the ratio of 1:9:9 (solution: methanol: acetonitrile; v/v). All solutions were vortexed and centrifuged at 15,500 rpm for 15 min. The supernatants were transferred to another tube and evaporated to dryness under a steady stream of air at room temperature. The residue was reconstituted with 80 µl of 50% aqueous methanol solution and centrifuged again at 15,500 rpm for 15 min. After centrifugation, 10 µl of the supernatant was injected to UPLC-MS/MS system for analysis. The density of the blood is treated as 1g/mL in the tissue distribution study.

5.3.9. UPLC-MS/MS Analysis of irinotecan, SN-38 and SN-38 glucuronide

The chromatographic separation of irinotecan, SN-38 and SN-38 glucuronide was achieved by a Waters Acquity™ UPLC H-Class system with a diode array detector (DAD) and a flow-through-needle sample manager. Analysis was carried out using Acquity UPLC BEH C18 Column (2.1 mm × 50 mm, 300˚A, 1.7 µm, Waters, Milford, MA, USA). Mobile phase A (0.1% formic acid in water [v/v]) and mobile phase B (100% acetonitrile) were operated with a gradient elution at a flow rate of 0.4 ml/min as follows: 10% B → 25% B (0 – 0.5 min), 25% B → 40% B (0.5 - 1 min), 40% B (1 – 2.5 min), 40% B → 10% B (2.5 – 4.5 min). The column temperature and sample temperature was 60°C and 20°C. The injection volume was 10 µl. 100 nM camptothecin (CPT) in 50% methanol-water was used as internal standard (IS). LC-MS/MS analysis for irinotecan, SN-38 and SN-38 glucuronide was performed with an API 5500 Qtrap triple quadruple

mass spectrometer coupled with a TurboIonSpray™ (Applied Biosystem- MDS SCIEX, Framingham, MA, USA). The system was operated in positive electrospray ionization (ESI) and multiple reactions monitoring (MRM) scan mode. All data were acquired and processed using Analyst®1.5.2 software with hotfixes (AB SCIEX). The main working parameters for mass spectrum were used in the QTRAP 5500 system as follows: ion-spray voltage, 5.5 kV; temperature, 500°C; curtain gas, 20 psig; gas 1, 20 psig; gas 2; 20 psig, collision gas, medium. The quantification was performed using MRM method and compound-dependent parameters in MRM mode for irinotecan, SN-38, SN-38 glucuronide and CPT were summarized in Table S1 (Supplemental Information).

5.3.10. Statistical Analysis

One way ANOVA with dunnet multiple comparison posttests was used to analyze the data of liver and kidney accumulation profile of irinotecan, SN-38 and SN-38 glucuronide in dose response and chemical inhibition study. Two-way analysis of variance test with bonferoni posttest (Graphpad prism) was used to analyze the data of the biliary, intestinal, urinary and plasma profile of irinotecan, SN-38 and SN-38 glucuronide in dose response and chemical inhibition study. The prior level of significance was set at 5%, or $p < 0.05$. In this article, '*'denotes p value less than 0.05, '**'denotes p value less than 0.01 and '***'denotes p value less than 0.001.

5.4. Results

5.4.1. Inhibition study of disposition of irinotecan, SN-38 and SN-38 glucuronide by chemical and Chinese herbal formulations using modified *in situ* rat perfusion model:

In this particular study, 5 mg/kg intravenous irinotecan was selected as control dose which is the equivalent dose of irinotecan demonstrating intestinal toxicity in human. As we already conducted inhibition study using 5 mg/kg cimetidine, so here we have presented same data to make a clear comparison on the efficacy of chemical and herbal treatment in terms of decreasing biliary and intestinal excretion of SN-38 and SN-38 glucuronide. As the active components of XCHT and PHY 906 have poor solubility, so we perfused both herbal products by after dissolving in HBSS buffer.

5.4.1.1 Biliary excretion rate and cumulative biliary excretion profile of irinotecan, SN-38 and SN-38 glucuronide:

Figure 24 represented the biliary excretion rate (panel a to c) and cumulative biliary excretion (panel d to f) profile of irinotecan, SN-38 and SN-38 glucuronide after co-administration of cimetidine and herbal products (XCHT and PHY 906) using *in situ* modified intestinal rat perfusion model. Biliary excretion rate profile (fig 24 a to c) suggested that XCHT has the ability to decrease irinotecan, SN-38 and SN-38 glucuronide at multiple time points spanning from 90 to 150 minutes. Similarly, PHY 906 has shown significant inhibitory effect on the biliary excretion of irinotecan and SN-38 glucuronide, although it doesn't have any effect on biliary excretion of SN-38. Cumulative biliary excretion profile of irinotecan (fig 24d) suggested that herbal formulation like XCHT (49%) and PHY 906 (51%) exhibited moderate inhibition on the

intestinal excretion of irinotecan, whereas cimetidine has mild effect (32%). In case of SN-38 (fig 24e), XCHT (62%) displayed comparatively better inhibitory effect of biliary excretion of SN-38 than cimetidine (41%). However, PHY 906 (61%) demonstrated strong inhibition on biliary excretion of SN-38 glucuronide, whereas both cimetidine (10%) and XCHT (35%) showed mild to moderate inhibition.

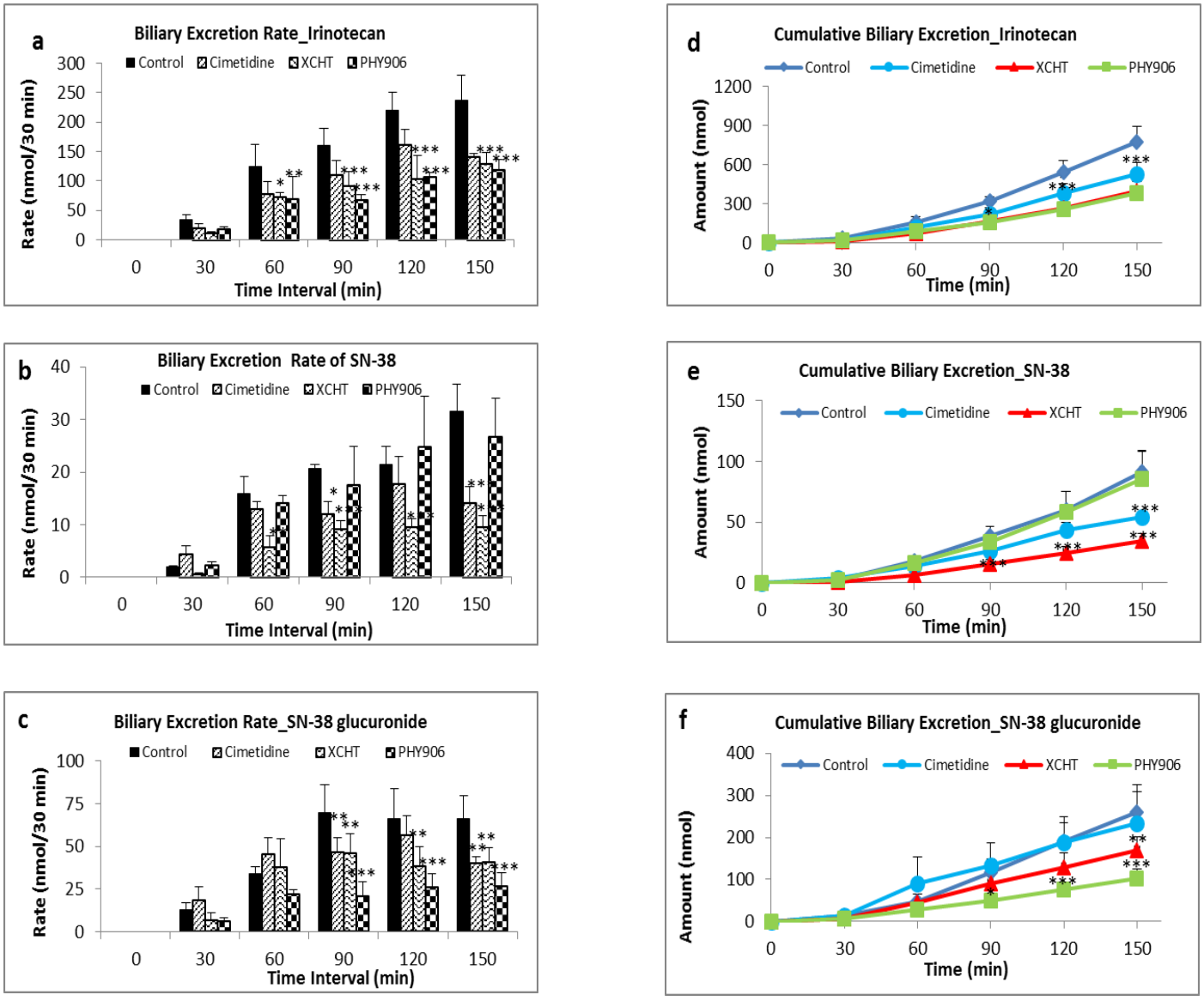


Figure 26: Comparison of the biliary excretion rate and cumulative biliary excretion profiles of irinotecan, SN-38 and SN-38 glucuronide after using cimetidine, XCHT and PHY 906 (n=4).

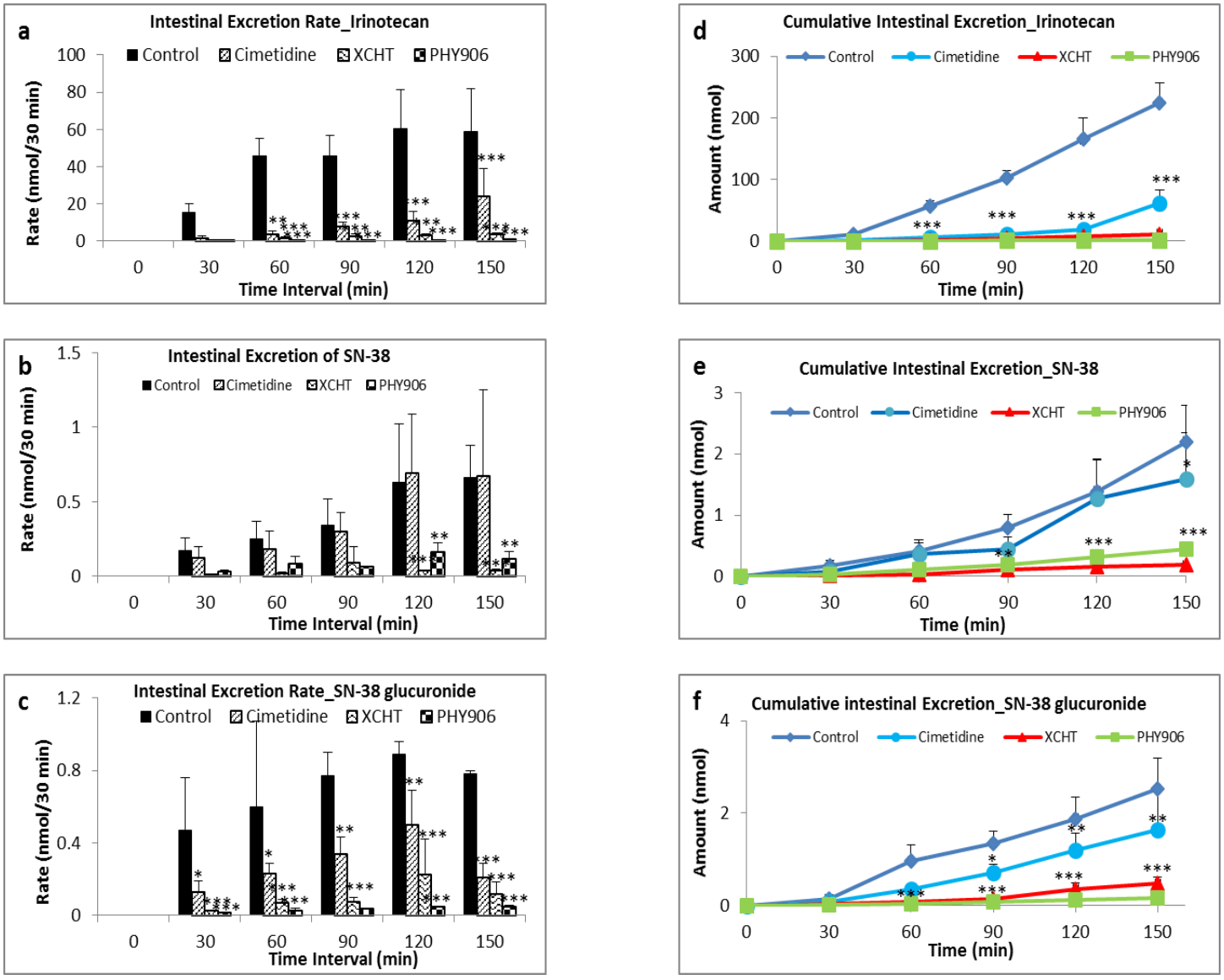


Figure 27: Comparison of the intestinal excretion rate and cumulative intestinal excretion profiles of irinotecan, SN-38 and SN-38 glucuronide after using cimetidine, XCHT and PHY 906 (n=4).

5.4.1.2 Intestinal excretion rate and cumulative intestinal excretion profile of irinotecan, SN-38 and SN-38 glucuronide:

Intestinal excretion rate profile (fig 25a) displayed that all the three compounds have shown drastic inhibition of intestinal excretion of irinotecan which was also reflected in the cumulative intestinal excretion plot of irinotecan (fig 25d). We found that all the compounds displayed strong inhibition ranging from approximately 70-100%. Contrary to the biliary excretion of SN-38, PHY 906 was found to decrease intestinal excretion of SN-38 significantly. In addition, all three compounds were found to decrease the intestinal excretion of SN-38 glucuronide. Cumulative intestinal excretion of SN-38 (fig 25e) and SN-38 glucuronide (fig 25f) demonstrated that both herbal formulations exhibited strong inhibitory effect on the intestinal excretion of SN-38 and SN-38 glucuronide ranging from 80-95%, whereas cimetidine showed relatively mild inhibition (28-35%).

5.4.1.3 Urinary excretion rate and cumulative urinary excretion profile of irinotecan, SN-38 and SN-38 glucuronide:

One of the advantages of our *in situ* modified rat perfusion model is the provision of determining the urinary excretion profile of test drug simultaneously with biliary and intestinal excretion profile in a single experiment. The aim of monitoring the urine profile was to investigate if the inhibitors have any significant impact on the urinary excretion of irinotecan and its metabolites during the whole operation. Both urinary excretion rate (fig 26 a to c) and cumulative urinary excretion (fig 26 d to f) profile demonstrated that none of the inhibitor have any kind of inhibitory effect of urinary excretion of SN-38 and SN-38

glucuronide. However, all three inhibitors exhibited moderate to strong inhibition of cumulative urinary excretion of irinotecan (63 to 79%).

5.4.1.4 Plasma profile of irinotecan, SN-38 and SN-38 glucuronide:

Plasma profile of irinotecan, SN-38 and SN-38 glucuronide described that none of the inhibitor altered the plasma profile of irinotecan significantly after treating with cimetidine, XCHT and PHY 906. Table 13 compared the AUC values of irinotecan, SN-38 and SN-38 glucuronide between control and treatment groups. Unlike cimetidine, both herbal products didn't alter the plasma profile of irinotecan. However, XCHT have a clear impact on the plasma profile of SN-38 where it increased the AUC value by 2 fold.

5.4.1.5 Bio-distribution of irinotecan, SN-38 and SN-38 glucuronide in liver and kidney:

Bio-distribution study of liver and kidney (fig 28) indicated that the accumulation of irinotecan and SN-38 is quite higher in liver in comparison to kidney in both control and treatment group. Though both cimetidine and PHY 906 can decrease the accumulation of irinotecan in liver by 58% and 14%, XCHT increases about 42% accumulation of irinotecan. However, in case of SN-38, all the compounds decreased approximately 16 to 55% accumulation of SN-38 in liver. On the other hand, all three compounds decreased 10 to 65% of irinotecan accumulation in kidney whereas only XCHT reduced accumulation of SN-38 by 58%. Figure 5 also demonstrated that both irinotecan (2 to 16 fold) and SN-38 (2 to 7 fold) accumulated preferentially in liver compared to kidney in both control and treatment groups. Lastly, this bio-distribution study also displayed that

SN-38 glucuronide was preferentially accumulated in kidney (2 to 4 fold) compared to liver in both control and treatment groups except XCHT.

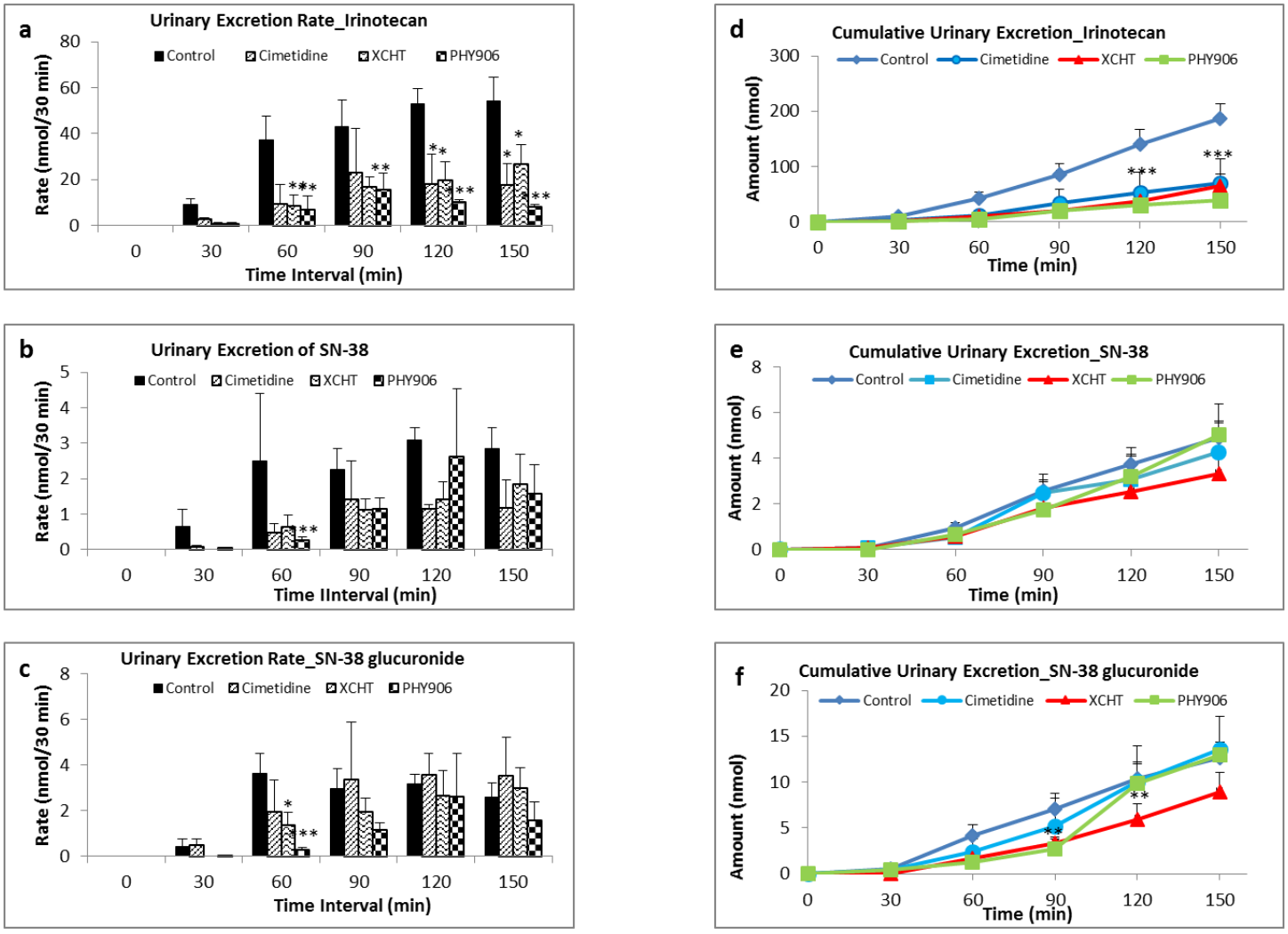


Figure 28: Comparison of the urinary excretion rate and cumulative urinary excretion profiles of irinotecan, SN-38 and SN-38 glucuronide after using cimetidine, XCHT and PHY 906 (n=4).

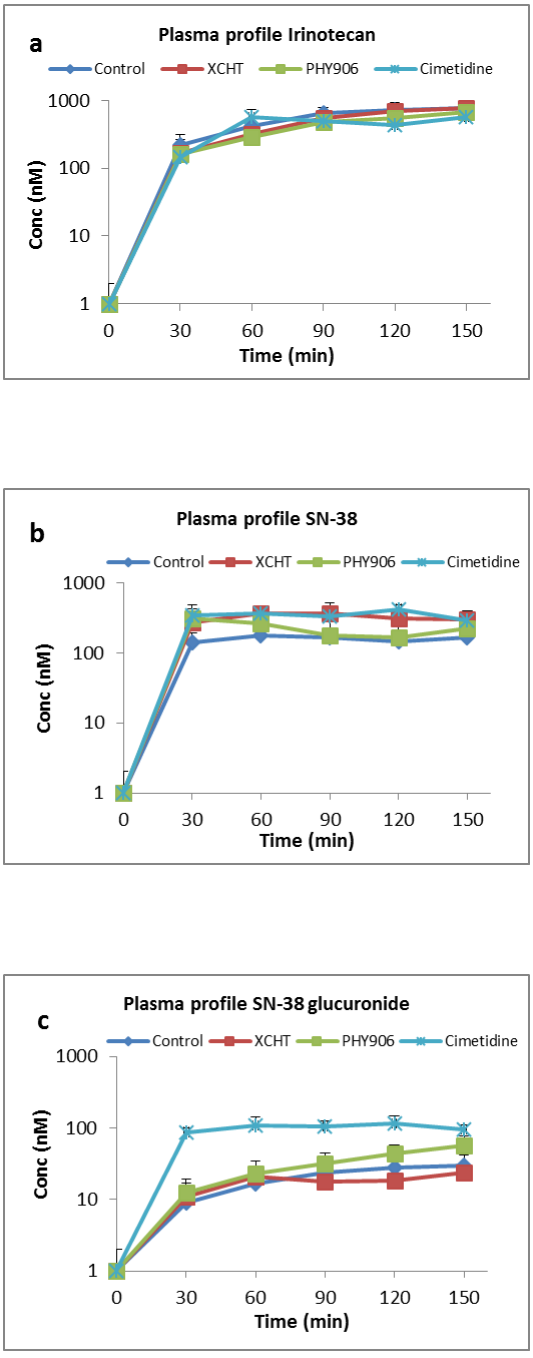


Figure 29: Comparison of the plasma profiles of irinotecan, SN-38 and SN-38 glucuronide after using cimetidine, XCHT and PHY 906 (n=4).

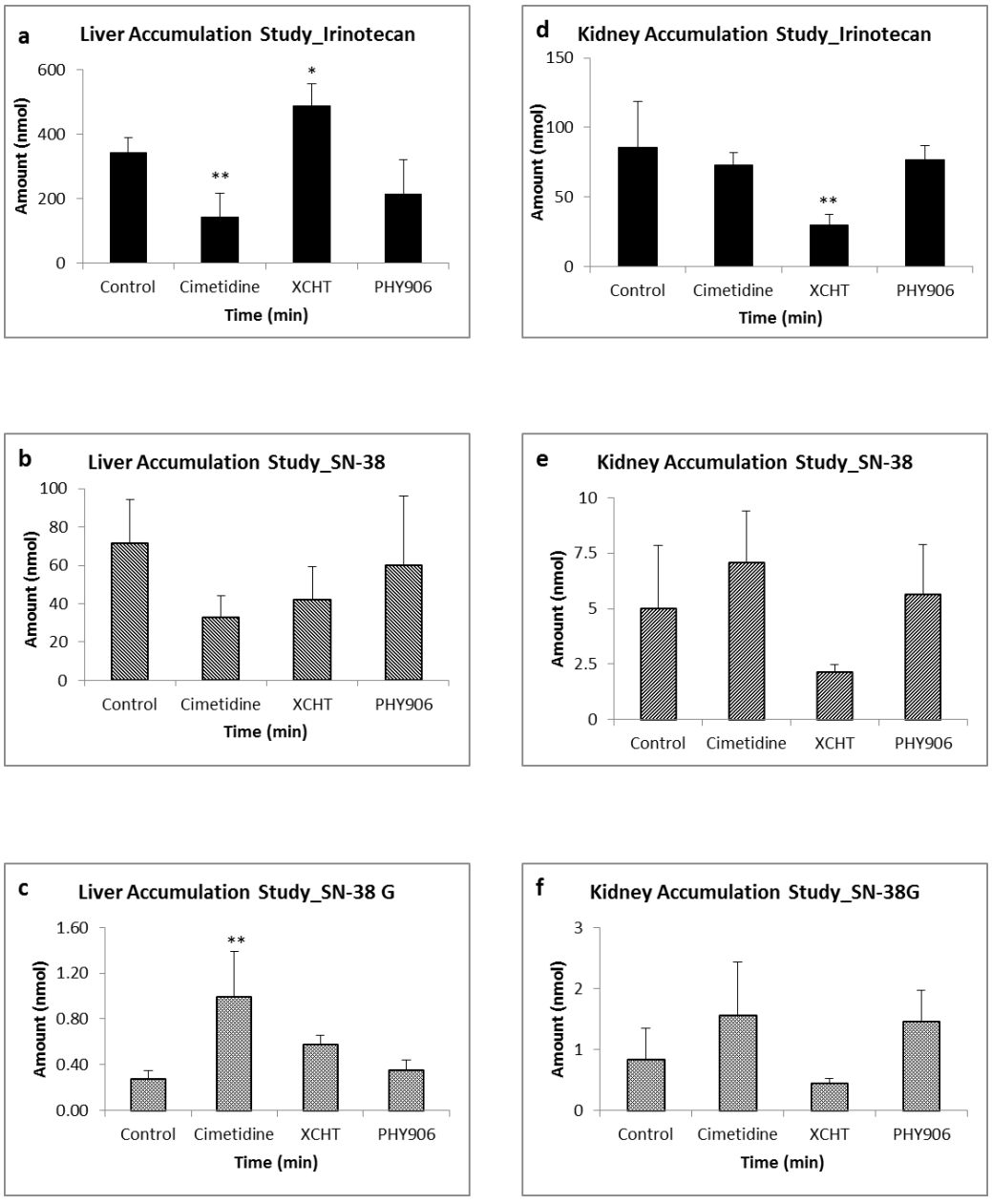


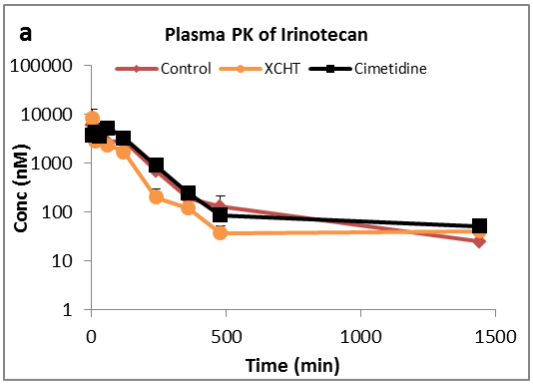
Figure 30: Comparison of the liver accumulation and kidney accumulation study of irinotecan, SN-38 and SN-38 glucuronide after using cimetidine, XCHT and PHY 906 (n=4)

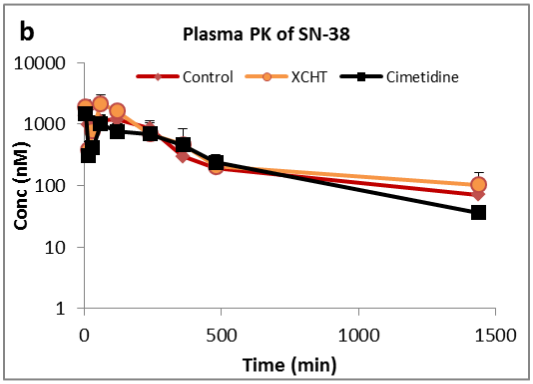
5.4.2. Effect of chemical inhibitor and herbal formulation on the disposition of irinotecan using *in vivo* rat pharmacokinetic study:

The results of our modified *in situ* rat perfusion study indicated that among all chemical inhibitors and herbal formulations, cimetidine and XCHT, displayed maximal inhibitory effect on the biliary and intestinal excretion of SN-38 and SN-38 glucuronide. Based on these observations, we conducted an *in vivo* rat pharmacokinetic study of irinotecan (5 mg/kg, iv) and coadministered cimetidine (5 mg/kg) and XCHT containing 100 µM of baicalin to determine if both of these drugs can decrease colonic SN-38 content *in vivo*. For this purpose, we collected blood (at different time points), urine (at 4, 8 and 24 hour) and feces (24 hour).

5.4.2.1 Effect of inhibitors on the plasma profile of irinotecan, SN-38 and SN-38 glucuronide:

Figure 29 displayed the plasma profile of irinotecan, SN-38 and SN-38 glucuronide after intravenous administration of irinotecan alone and with inhibitors. By analyzing the plasma profile, we found out that none of these inhibitors altered the plasma concentration of SN-38 which implies that there is no alteration of efficacy of the irinotecan after treating with cimetidine and XCHT. Similarly, the plasma profile of SN-38 glucuronide also described that none of these inhibitor have any effect of the plasma profile of SN-38 glucuronide.





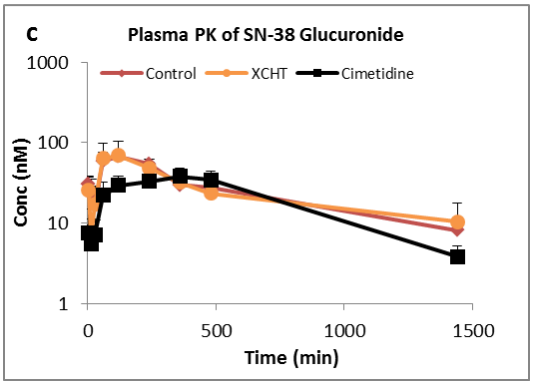


Figure 31: Comparison of the plasma profile of irinotecan, SN-38 and SN-38 glucuronide in pharmacokinetic study after using cimetidine and PHY 906 (n=6)

5.4.2.2 Effect of inhibitors on the fecal excretion profile of irinotecan, SN-38 and SN-38 glucuronide:

Figure 30 demonstrated the fecal excretion profile of irinotecan, SN-38 and SN-38 glucuronide at 24 hours. The fecal excretion profile of irinotecan and SN-38 displayed that XCHT can decrease the fecal content of both these compounds by 50% and 42%, respectively. On the other hand, cimetidine can only decrease the fecal content of SN-38 by 46%. However, both of these compounds don't affect the fecal content of SN-38 glucuronide.

5.4.2.3 Effect of inhibitors on the urinary excretion profile of irinotecan, SN-38 and SN-38 glucuronide:

Contrary to fecal excretion of irinotecan and SN-38, both cimetidine and XCHT didn't exhibit any significant effect on the urinary excretion of irinotecan and SN-38 at multiple time points ranging from 4 to 24 hours. We haven't observed any urine after 4 hour of treatment of cimetidine which is denoted as '*'.

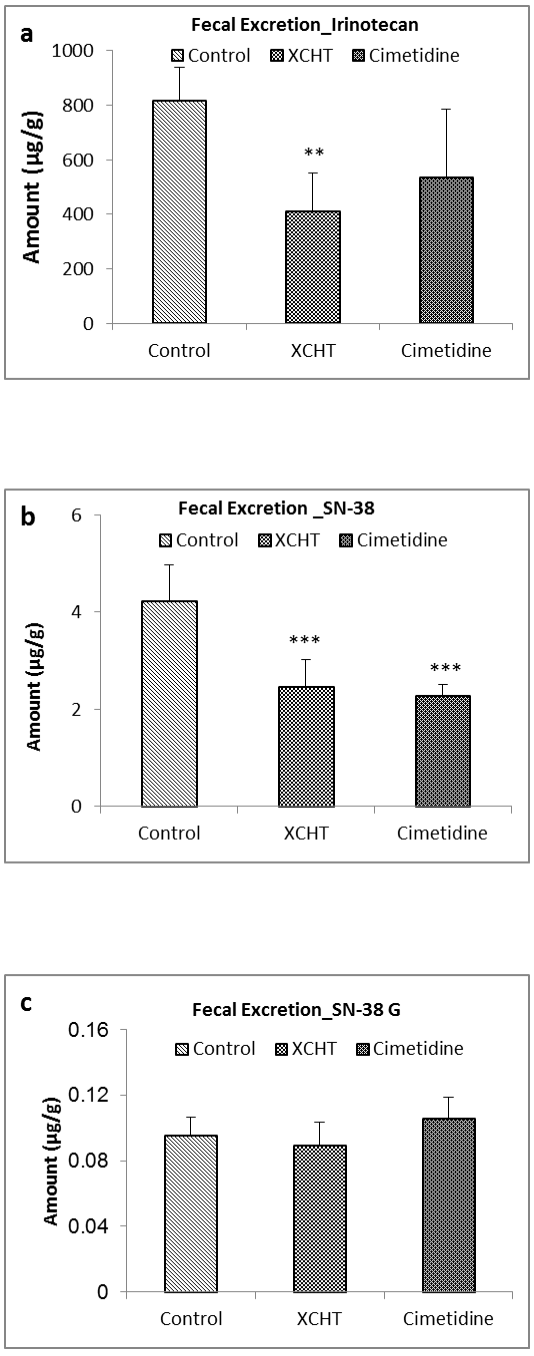
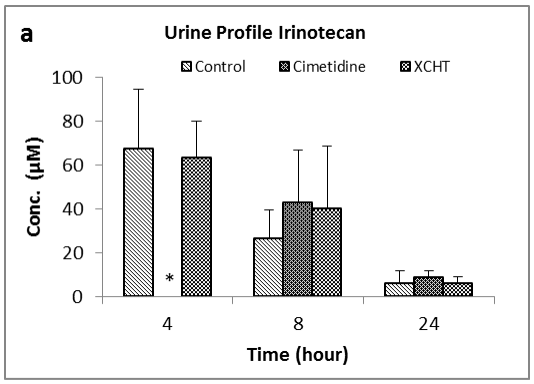
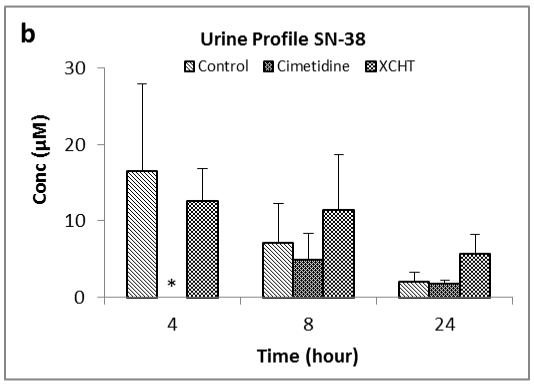


Figure 32: Comparison of the fecal excretion profile of irinotecan, SN-38 and SN-38 glucuronide in pharmacokinetic study after using cimetidine and PHY 906 (n=6)





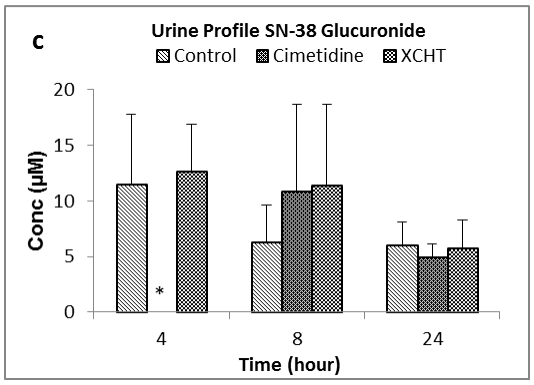


Figure 33: Comparison of the urinary excretion profile of irinotecan, SN-38 and SN-38 glucuronide in pharmacokinetic study after using cimetidine and PHY 906 (n=6)

5.5. Discussion

Late onset diarrhea induced by irinotecan is one of the major problems which limit the usage of irinotecan in approximately 10-20% of patients receiving chemotherapy (Mathijssen, van Alphen et al. 2001; Yang, Hu et al. 2005). Mechanistic studies revealed that accumulation of SN-38 in intestinal lumen causes late onset diarrhea as SN-38 has the ability to damage intestinal epithelium (Sugiyama, Kato et al. 1998; Ma and McLeod 2003). It has been observed that combination of different factors such as activation of SN-38 from irinotecan by carboxylesterase, regeneration of SN-38 from inactive SN-38 glucuronide by bacterial β-glucuronidase, excessive biliary and intestinal excretion of SN-38 through efflux transporters etc. lead to elevate SN-38 content in intestinal lumen ultimately resulting in late onset diarrhea (Gupta, Lestingi et al. 1994; Catimel, Chabot et al. 1995; Takasuna, Hagiwara et al. 1996; Chu, Kato et al. 1997; Ahmed, Vyas et al. 1999; Smith, Figg et al. 2006). Till date, several approaches have been taken to reduce the incidence of diarrhea by modulating the activity of enzymes and efflux transporters, but none of them were able to be clinically effective (Gupta, Safa et al. 1996; Horikawa, Kato et al. 2002). In this particular article, using our *in situ* modified rat perfusion model we demonstrated that one chemical inhibitor cimetidine and one Chinese herbal formula XCHT (known as SST or TJ-9 in Japan) are capable of reducing the biliary and intestinal excretion of SN-38 and SN-38 glucuronide which might lead to amelioration of irinotecan induced late onset diarrhea. Moreover, *in vivo* rat pharmacokinetic study suggested that both these inhibitors are capable to reduce the fecal content of SN-38 without altering the plasma profile of SN-38 which is responsible of exerting anticancer action.

Herbal medicines have been commonly used by cancer patients in Asia to combat various diseases (Zhang, Saif et al. 2010; Liu and Cheng 2012). In case of irinotecan induced chemotherapy, different herbs already have been tried to reduce the severity of irinotecan associated diarrhea. Animal experiments have shown that Kampo medicine Hangeshashin-to (TJ-14) can effectively prevent irinotecan associated late diarrhea on rat model (Katoh, Yoshioka et al. 2009; Chikakiyo, Shimada et al. 2012). Presence of baicalin, which is a bacterial β-glucuronidase inhibitor, has been found to be responsible for its inhibitory action. Though preclinical studies indicated the protective action of TJ-14 against irinotecan associated diarrhea, clinical trials involving non-small cell lung cancer patients reported no significant difference in the overall frequency or duration of diarrhea (Kummar, Copur et al. 2011). Apart from inhibition of bacterial β glucuronidase, baicalin is also a good substrate of efflux transporter MRP-2 (Akao, Sato et al. 2009). So co-administration of herbal products containing high amount of baicalin might reduce the biliary excretion of SN-38 and SN-38 glucuronide by competitive inhibiting the activity of MRP2 towards SN-38 and SN-38 glucuronide. Due to inhibitory property of baicalin towards bacterial β glucuronidase and MRP2 activity in liver, concomitant administration of high amount of baicalin containing herbal formulation might be a viable option to alleviate irinotecan induced late onset diarrhea by reducing the SN-38 content in intestinal lumen.

In this particular study we have selected two different Chinese herbal formulations such as PHY 906 and Xiaochaohutang (XCHT) which can be used to prevent irinotecan associated diarrhea. Among them, PHY 906 is relatively well studied herbal formulation

which has been used extensively for the treatment of common gastrointestinal distress including diarrhea, abdominal spasms, fever, headache etc. (Liu and Cheng 2012). It has also been utilized to treat diarrhea associated with irinotecan treatment in both preclinical and clinical studies. Preclinical studies indicated that PHY 906 was able to improve the condition of irinotecan treated patient by decreasing irinotecan associated body weight loss and mortality rate (Liu and Cheng 2012). Mechanistically it has been proved that PHY 906 doesn't have any significant effect on the activation of SN-38 by bacterial β glucuronidase as it did not protect the onset of irinotecan induced damage to the intestine. On the other hand, the protective action of PHY 906 is attributed to its ability to restore the intestinal epithelium by promoting the regeneration of intestinal progenitor/stem cells. Our *in situ* modified rat perfusion study also supported the fact where we observed that there is no significant inhibition of biliary excretion of SN-38 after PHY 906 treatment although it demonstrated strong inhibition of intestinal excretion of SN-38.

XCHT, like PHY 906, is a Chinese herbal formulation which contains baicalin. Due to the inhibition property of baicalin, we perfused 100 μM of baicalin containing XCHT alongwith 5mg/kg irinotecan using our *in situ* modified rat perfusion model. Contrary to PHY 906, we found that XCHT has the ability to decrease both biliary and intestinal excretion by large extent (from 60 to 90%). Apart from SN-38, this particular herb can also decrease the intestinal and biliary excretion of SN-38 glucuronide (35 to 80%). Though we didn't conduct any systematic investigation to determine the actual reason of the inhibitory action demonstrated by XCHT, we can speculate that presence of high

amount of baicalin might be one of the important reason. However, after comparing the baicalin concentration from control group (XCHT perfused group only) and treatment group (irinotecan and XCHT) in a separate experiment, we found out that the biliary excretion of baicalin was increased by 4 fold (data unpublished). This particular observation obviously states the importance of presence of baicalin in the Chinese herbal formulation.

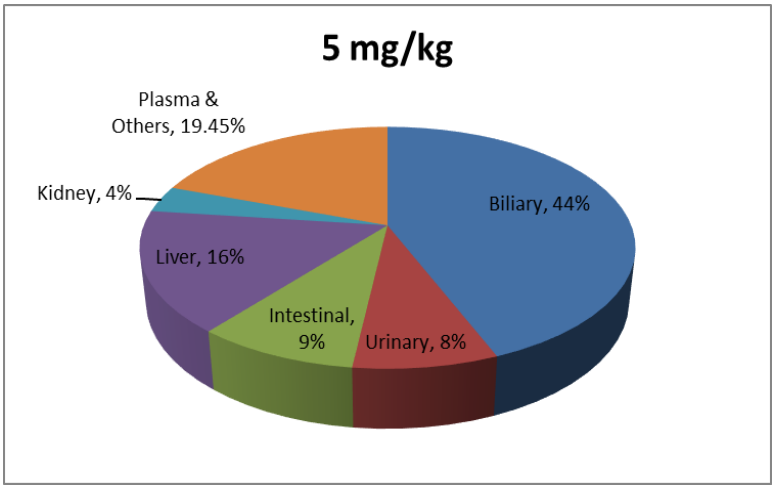
Chemical inhibition study of irinotecan suggested that (Chapter 5) among all the chemical inhibitors cimetidine is the only drug which showed significant inhibition of biliary excretion of SN-38 and SN-38 glucuronide. In terms of inhibition, XCHT showed better inhibitory profile compared to cimetidine both in bile (37% higher) and intestinal perfusate (88% higher). As both of them showed moderate to strong inhibition of SN-38 in both biliary and intestinal excretion, we included them in the *in vivo* rat pharmacokinetic study to observe if they can translate this particular activity *in vivo*. Fecal excretion profile of SN-38 and SN-38 glucuronide suggested that XCHT can decrease the fecal content of irinotecan and SN-38 by 46 to 50%; whereas cimetidine inhibited selectively the fecal content of SN-38 by 42%. This particular inhibitory action of XCHT also supported the fact that high concentration of baicalin inhibited the activity of bacterial β glucuronidase by competitive inhibition which has been pointed out in other studies (Narita, Nagai et al. 1993). Apart from that, another reason of the decreased fecal content of SN-38 after XCHT treatment might be the less formation of SN-38 glucuronide due to limited availability of UGT enzymes. Both baicalin and wogonoside after hydrolysis forms its corresponding aglycone baicalein and wogonin – which can be

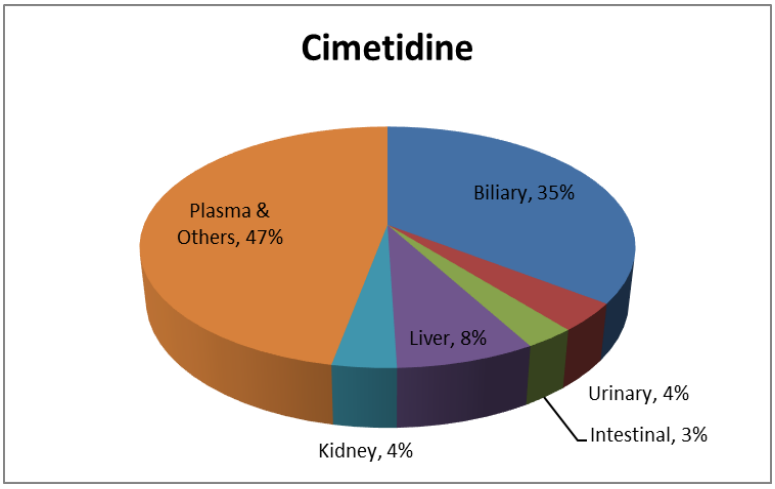
susceptible to glucuronidation in liver and excreted through bile ultimately reducing the availability of UGT enzymes competitively. 4 fold higher formation of baicalin supports the fact that baicalein as an aglycone form can competitively inhibit the formation of SN-38 glucuronide by UGT. However, additional mechanistic studies should be performed to support this fact.

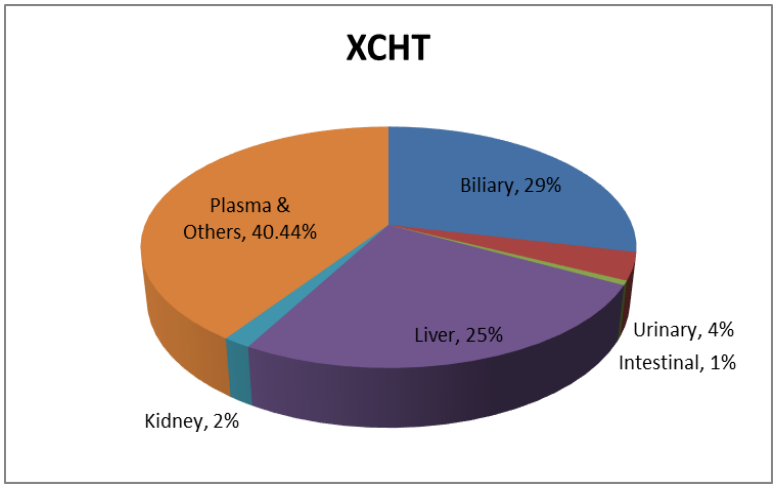
To the best of our knowledge, this is the first study where cimetidine and Chinese herb formulation XCHT have displayed their ability to decrease the biliary excretion of SN-38 and SN-38 glucuronide. Apart from biliary excretion, XCHT also decreased significantly the intestinal excretion of SN-38. In addition, we also demonstrated for the first time that cimetidine and XCHT can reduce the fecal excretion of SN-38 without altering its plasma profile in pharmacokinetic study. From all of these observations we can conclude that both of these agents might decrease the incidence of diarrhea induced by irinotecan as they decreased the colonic content of SN-38 by inhibiting fecal content of SN-38. However, both cimetidine and XCHT should be tested in a preclinical colon cancer toxicity model to show their effectiveness. In addition, further mechanistic studies are also warranted to unravel the inhibition mechanism displayed by XCHT.

In conclusion, our *in situ* modified rat perfusion model coupled with *in vivo* pharmacokinetic study demonstrated that the concomitant administration of chemical inhibitor cimetidine and traditional Chinese herbal formula XCHT can significantly inhibit the SN-38 content in intestinal lumen by decreasing biliary and intestinal excretion of SN-38 and SN-38 glucuronide *in vitro* and fecal excretion of SN-38 *in vivo*. The observed effect may be advantageous in terms of ameliorating irinotecan-associated diarrhea.

Moreover, we haven't observed any toxicity in the animal model due to XCHT usage. Further investigations are warranted to explore the underlying mechanisms for the observed herb-drug interactions and identification of key herbal ingredients present in XCHT responsible for the inhibitory effect. Lastly, these data are stimulating for further identification of herb–drug interactions which might be strategically applied to reduce toxicity of marketed drugs.







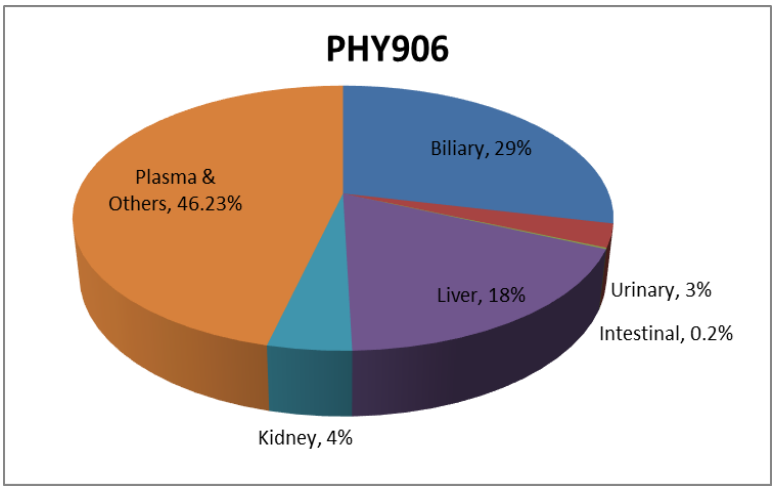


Figure 34: Mass balance study of irinotecan, SN-38 and SN-38 glucuronide after using cimetidine and herbal formulations (PHY 906 and XCHT)

Table 12: Percentage excretion of irinotecan, SN-38 and SN-38 glucuronide in Bile, Perfusate, Urine, Liver and Kidney after chemical inhibition study

Group / Route	Control			Cimetidine			XCHT			PHY 906		
	Irinotecan	SN-38	SN-38G	Irinotecan	SN-38	SN-38G	Irinotecan	SN-38	SN-38G	Irinotecan	SN-38	SN-38G
Bile (%)	30.24	3.57	10.12	22.87	2.34	10.07	18.89	1.64	8.07	19.18	4.3	5.13
Perfusate (%)	8.74	0.09	0.1	2.65	0.07	0.07	0.56	0.01	0.02	0.11	0.02	0.01
Urine (%)	7.27	0.19	0.49	3.01	0.18	0.59	3.08	0.16	0.43	1.92	0.25	0.65
Liver (%)	13.38	2.78	0.01	6.2	1.42	0.04	23.14	2.01	0.03	14.92	3.03	0.02
Kidney (%)	3.33	0.19	0.03	3.15	0.30	0.07	1.42	0.10	0.02	3.86	0.28	0.07

Table 13: AUC (±SD) (μM· min) of irinotecan, SN-38 and SN-38 glucuronide in plasma at 150 min after perfusion study

Group / Route	Control			Cimetidine			XCHT			PHY 906		
	Irinotecan	SN-38	SN-38G	Irinotecan	SN-38	SN-38G	Irinotecan	SN-38	SN-38G	Irinotecan	SN-38	SN-38G
Plasma	22.46 (2.46)	4.68 (1.2)	0.86 (0.37)	15.14 (6.76)	10.73 (1.46)	3.16 (1.39)	22.12 (6.8)	9.2 (2.32)	0.62 (0.11)	18.18 (1.4)	5.89 (2.58)	1.5 (0.54)

Summary

In this thesis work, we aim to investigate mechanisms that are responsible to reduce the colonic SN-38 exposure which will ultimately prevent the incidence of late onset diarrhea caused by irinotecan. To achieve this purpose, we developed a UPLC-MS/MS method for the simultaneous determination of irinotecan and its metabolites in different bio-matrices, established a modified *in situ* rat perfusion model to determine the impact of efflux transporters on the disposition of irinotecan and performed a rat pharmacokinetic study to evaluate the effectiveness of chemical and herbal agents to decrease the fecal excretion of SN-38.

We have demonstrated that

- Our developed and validated LC-MS/MS method can be applied to quantify irinotecan, SN-38 and SN-38 glucoronide simultaneously at different bio-matrices (plasma, urine, bile and intestinal perfusate) and metabolic organs (liver and kidney).
- Caco-2 cell culture study indicated that the transport of irinotecan and SN-38 is mediated by efflux transporters.
- Dose response study using the developed modified *in situ* rat perfusion model depicted that biliary route is the predominant excretion route for irinotecan, SN-38 and SN-38 glucuronide. The high steady state concentration of irinotecan and its metabolites in bile and urine compared to their plasma concentration

suggested that the excretion of these compounds was mainly governed by predominant efflux transporters.

- Chemical inhibition study using the same modified *in situ* rat perfusion model demonstrated that both cyclosporine A (18%) and cimetidine (41%) has mild to moderate inhibitory effect on the biliary excretion of SN-38; whereas cyclosporine A (45%) and quercetin (23%) can inhibit the biliary excretion of SN-38 glucuronide.
- All the chemicals except erlotinib can demonstrate moderate to strong inhibitory effect (28 to 96%) on the intestinal excretion of SN-38.
- We have demonstrated for the first time that MATE-1 transporter can also play a significant role on the disposition of irinotecan.
- Our modified *in situ* rat perfusion model may be utilized as a screening tool to identify the potential chemical or herbal agent which can inhibit biliary and intestinal excretion of SN-38 and SN-38 glucuronide.
- We have displayed for the first time that one traditional chinese medicine/herbal formulation Xiao Chao Hu Tang (XCHT/TJ-14) were able to demonstrate strong inhibition of biliary (60%) as well as intestinal (90%) excretion of both SN-38 and SN-38 glucuronide.
- *In vivo* rat pharmacokinetic study revealed that both cimetidine and Xiao Chao Hu Tang can reduce the fecal excretion of SN-38 which might lead to the alleviation of irinotecan induced late onset diarrhea. In addition, we also observed that the plasma profile of SN-38 wasn’t altered after chemical and

herbal treatment which means the efficacy of chemotherapeutic treatment was unaltered.

- Findings from perfusion and pharmacokinetic studies suggested that our developed modified *in situ* rat perfusion model may be utilized as a screening tool to strategically select chemical and herbal agents which can reduce drug associated toxicity by modulating efflux transporters

Appendix: Drug detoxifying bacteria

Background:

Irinotecan (CPT-11) is a highly effective anticancer drug which is widely utilized in first and second line treatment of metastatic colon cancers, typically in combination with other agents (Saltz 1997, Mathijssen et al., 2001). However the use of Irinotecan is often dose-limited and possibly efficacy-limited as it causes severe late-onset diarrhea in approximately 30% of the patient population (Rothenberg et al., 1996, Kurita et al., 2011). Experimental studies have revealed a variety of pathological changes in the intestines of irinotecan treated animals including increased apoptosis of crypt cells in the duodenum, jejunum and colon, loss of brush-border enzymes in all regions of the intestine, and scanning electron microscopy documentation of villus shortening and loss in the ileum (Nagar and Blanchard 2006). Therefore the reduction of the intestinal toxicity of Irinotecan via non-invasive means will greatly improve its therapeutic outcomes.

Recent studies indicate the irreversible formation of a toxic metabolite of Irinotecan in the gut known as SN-38 to be the major cause of the drug-induced intestinal toxicity leading to diarrhea (Araki et al., 1993, Mathijssen et al., 2001). Following its intravenous administration, Irinotecan is metabolically activated in the liver to SN-38. Subsequently SN-38 is inactivated by glucuronidation and forms SN-38 glucuronide (SN-38G) which is excreted via bile. However within the gut, SN-38G is deconjugated irreversibly to SN-38 by bacterial β-glucuronidase enzymes secreted from the commensal microbiota that reside in the intestine and causes diarrhea (Stein, Voigt et al. 2010). Several approaches already have been taken to circumvent SN38 mediated late onset diarrhea such as usage of antibiotics (neomycin and streptomycin), alkaline agents/pH modifier (sodium bicarbonate), and synthesis of bacterial β-glucuronidase inhibitors; but none of these is clinically utilized.

Approach:

From preliminary results we observed purified several purified plant UDP-glucuronyl transferase (pUGT) isoforms can glycosylate SN-38. Based on this observation, we hypothesize that an engineered bacterium which overexpresses a plant UGT enzyme can reduce SN-38 content in colon after glycosylating SN-38 to form inactive SN-38 glucose. As there is a direct correlation between SN-38 content in colon and the event of diarrhea, so we can assume reduction of colonic content of SN-38 by pUGT expressed E. coli will circumvent the incidence of diarrhea in irinotecan treated patients.

Results:

In vitro Kinetic study:

In vitro enzyme kinetic studies suggest that several pUGT isoforms can glycosylate SN-38 in presence of UDP glucose. We have used six purified pUGT enzymes (UGT71G1, UGT81G1, UGT85H2, GT22D, GT22E and UGT78G1) to perform glycosylation experiment of SN-38. Among them, UGT71G1, UGT78G1 and GT22D were able to glycosylate SN-38 (Fig 32).

In vitro Cell based Glycosylation study:

Based on these results we expressed those pUGT isoforms in E. coli [BL21 (DE3) strain] and performed *in vitro* glycosylation study of Irinotecan and its active metabolite SN-38 with 10^{10} cfu/ml pUGT expressed E. coli in presence of UDP glucose. From the cellular experiments we observed only pUGT71G1 expressed E. coli has the ability to glycosylate 5 µM SN-38. After 2 h incubation, 8.1-20% free drug (SN-38) was converted to its glucoside form at doses of 0.25-5 µM SN-38, and this number can reach > 80% when the incubation time was prolonged (Fig. 33).

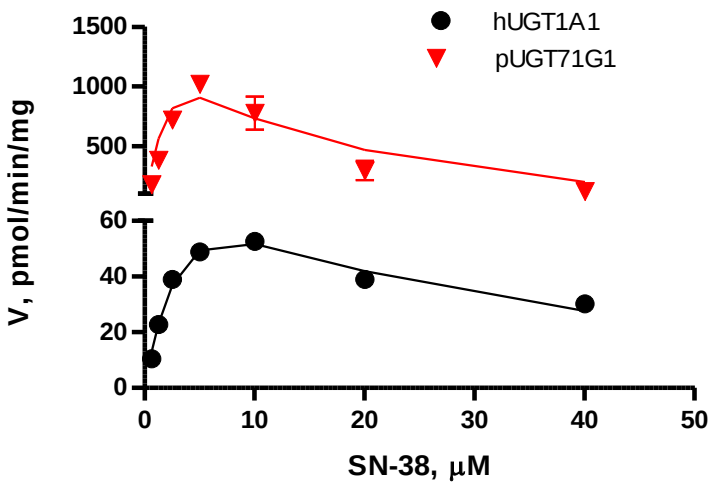


Figure 35: *In vitro* kinetic experiment of SN-38 by purified human enzymeUGT1A1 and plant UGT71G1.

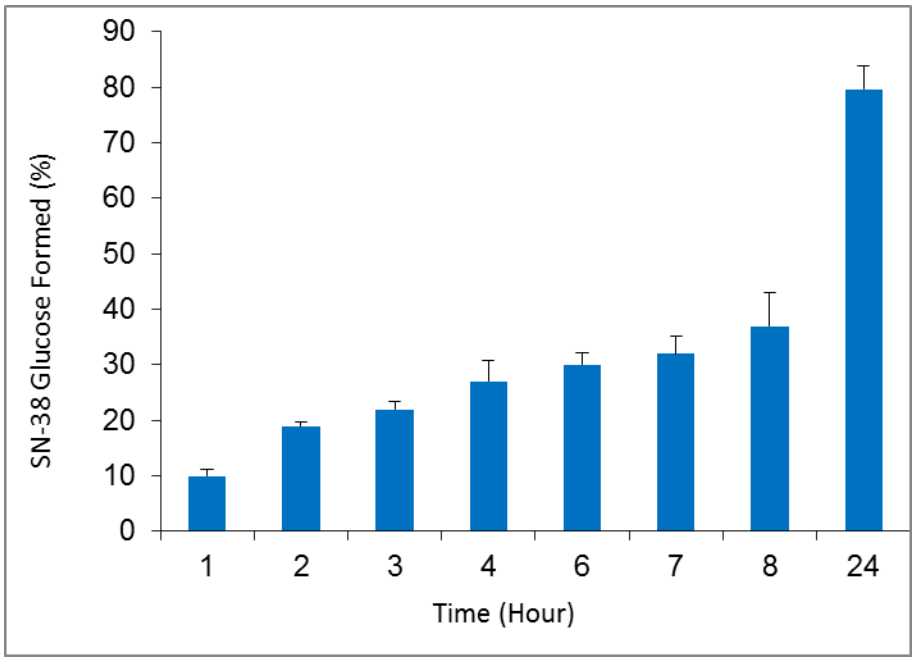


Figure 36: *In vitro* cell based glycosylation experiment of SN-38 by pUGT71G1 in presence of UDP-glucose.

In vivo Pharmacokinetic study:

For *in vivo* pharmacokinetic study we administered plant UGT expressed E.coli to Balb/c mice (body weight 20 -25 gm) orally for three consecutive days and on the fourth day we administered SN38 intraperitoneally or orally. Before treating with bacteria, we pretreated the animals with omeprazole, vancomycin and imipenam (each 50 mg/kg). After treating the animals with 10mg/kg SN38 i.p., we collected blood from tail vein at 0, 15, 30, 60, 120, 180, 240, 480 and 1440 minutes and the feces after 6 and 24 hour. For oral administration, we gavaged SN38 at 2.5 mg/kg daily and collect blood and feces after 6 hours of dosing. We sacrificed the animals after 3 days dosing and collected blood and feces as well as preserved colon and intestine at -80˚F. After oral and i.p. administration of SN-38, we could not observe formation of SN-38 glucose in blood and feces.

MTT Assay:

We performed MTT cell proliferation assay to measure the cellviability after treating the cells with SN-38 and SN-38 glucose. We incubated different concentrations of SN-38 and SN-38 glucose ranging from 0.3125 µM to 10 µM with cells for 4 hours and measured the absorbance values using spectrophotometer. We found after the treatment of SN-38, only 80% cells can survive whereas SN-38 glucose didn't affect the cell viability at all.

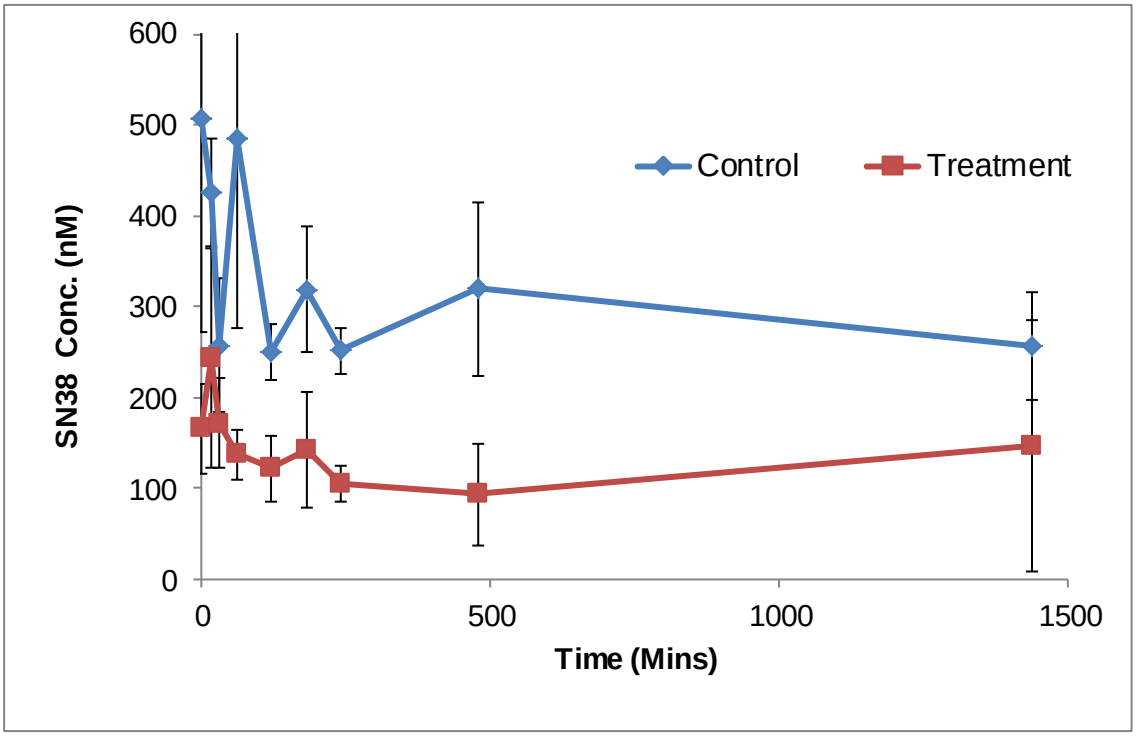


Figure 37: Plasma conc. of SN-38 after administration of 10 mg/kg SN-38 i.p. with 10^{10} cfu/ml pUGT71G1 expressed E. coli.

Problems:

The main problem of this approach was the instability of SN-38 glucose. We developed a UPLC-MS/MS method to quantify irinotecan, SN-38 and SN-38 glucose simultaneously in different bio-matrices like plasma, feces and urine. But we found the recovery of SN-38 glucose in feces is extremely low about 10%. After heating the fecal matrix, this value reaches to its maximum value around 30%. However, though the recovery of the drug in plasma was moderate (60 to 75%), after pharmacokinetic study we couldn't find the formation of SN-38 glucose either in blood or in feces. To explain this problem we can speculate multiple factors such as low recovery of SN-38 glucose in feces, instability of SN-

38 glucose in blood, loss of potency of enzymes, presence of insufficient amount of bacteria in the body may be the reason for absence of SN-38 glucose in the blood and feces.

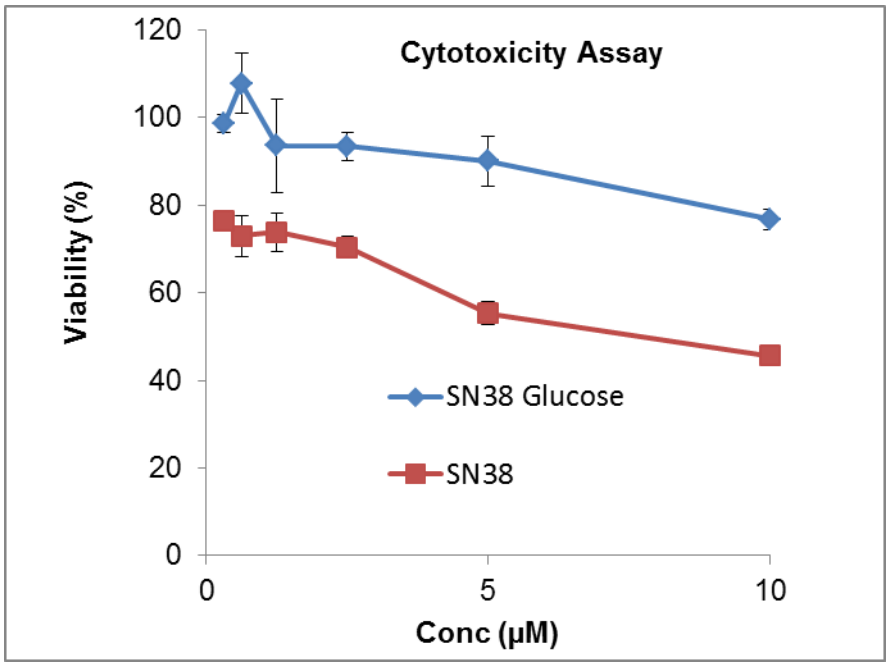


Figure 38: MTT Assay to compare cytotoxicity of SN-38 and SN-38 glucose (Incubating Conc. 0.3125 µM- 10 µM).

References

Abigerges, D., G. G. Chabot, et al. (1995). "Phase I and pharmacologic studies of the camptothecin analog irinotecan administered every 3 weeks in cancer patients." J Clin Oncol **13**(1): 210-221.

Ahmed, F., V. Vyas, et al. (1999). "*In vitro* activation of irinotecan to SN-38 by human liver and intestine." Anticancer Res **19**(3A): 2067-2071.

Ahn, G., D. M. Park, et al. (2014). "A rapid, simple and reliable HPLC-triple quadrupole tandem mass spectrometer method for a simultaneous quantification of irinotecan and its active metabolite 7-ethyl-10-hydroxycamptothecin (SN38) in mouse plasma." Biomed Chromatogr **28**(7): 919-922.

Akao, T., K. Sato, et al. (2009). "Hepatic contribution to a marked increase in the plasma concentration of baicalin after oral administration of its aglycone, baicalein, in multidrug resistance-associated protein 2-deficient rat." Biol Pharm Bull **32**(12): 2079-2082.

Akhtar, R., S. Chandel, et al. (2014). "Current status of pharmacological treatment of colorectal cancer." World J Gastrointest Oncol **6**(6): 177-183.

Araki, E., M. Ishikawa, et al. (1993). "Relationship between development of diarrhea and the concentration of SN-38, an active metabolite of CPT-11, in the intestine and the blood plasma of athymic mice following intraperitoneal administration of CPT-11." Jpn J Cancer Res **84**(6): 697-702.

Arimori, K., N. Kuroki, et al. (2003). "Effect of P-glycoprotein modulator, cyclosporin A, on the gastrointestinal excretion of irinotecan and its metabolite SN-38 in rats." Pharm Res **20**(6): 910-917.

Arimori, K., N. Kuroki, et al. (2001). "Excretion into gastrointestinal tract of irinotecan lactone and carboxylate forms and their pharmacodynamics in rodents." Pharm Res **18**(6): 814-822.

Armand, J. P. (1996). "CPT-11: clinical experience in phase I studies." Semin Oncol **23**(1 Suppl 3): 27-33.

Azim, H. A., Jr. and A. Awada (2012). "Clinical development of new formulations of cytotoxics in solid tumors." Curr Opin Oncol **24**(3): 325-331.

Bansal, T., A. Awasthi, et al. (2008). "Pre-clinical evidence for altered absorption and biliary excretion of irinotecan (CPT-11) in combination with quercetin: possible contribution of P-glycoprotein." Life Sci **83**(7-8): 250-259.

Bansal, T., G. Mishra, et al. (2009). "Effect of P-glycoprotein inhibitor, verapamil, on oral bioavailability and pharmacokinetics of irinotecan in rats." Eur J Pharm Sci **36**(4-5): 580-590.

Bardin, S., W. Guo, et al. (2005). "Liquid chromatographic-tandem mass spectrometric assay for the simultaneous quantification of Camptosar and its metabolite SN-38 in mouse plasma and tissues." J Chromatogr A **1073**(1-2): 249-255.

Benson, A. B., 3rd, J. A. Ajani, et al. (2004). "Recommended guidelines for the treatment of cancer treatment-induced diarrhea." J Clin Oncol **22**(14): 2918-2926.

Bleiberg, H. and E. Cvitkovic (1996). "Characterisation and clinical management of CPT-11 (irinotecan)-induced adverse events: the European perspective." Eur J Cancer **32A Suppl 3**: S18-23.

Boyd, G., J. F. Smyth, et al. (2001). "High-performance liquid chromatographic technique for the simultaneous determination of lactone and hydroxy acid forms of camptothecin and SN-38 in tissue culture media and cancer cells." Anal Biochem **297**(1): 15-24.

Brandi, G., J. Dabard, et al. (2006). "Intestinal microflora and digestive toxicity of irinotecan in mice." Clin Cancer Res **12**(4): 1299-1307.

Burke, T. G. and Z. Mi (1994). "The structural basis of camptothecin interactions with human serum albumin: impact on drug stability." J Med Chem **37**(1): 40-46.

Catimel, G., G. G. Chabot, et al. (1995). "Phase I and pharmacokinetic study of irinotecan (CPT-11) administered daily for three consecutive days every three weeks in patients with advanced solid tumors." Ann Oncol **6**(2): 133-140.

Chabot, G. G. (1997). "Clinical pharmacokinetics of irinotecan." Clin Pharmacokinet **33**(4): 245-259.

Chen, J., H. Lin, et al. (2003). "Metabolism of flavonoids via enteric recycling: role of intestinal disposition." J Pharmacol Exp Ther **304**(3): 1228-1235.

Chen, S., M. F. Yueh, et al. (2013). "Intestinal glucuronidation protects against chemotherapy-induced toxicity by irinotecan (CPT-11)." Proc Natl Acad Sci U S A **110**(47): 19143-19148.

Chester, J. D., S. P. Joel, et al. (2003). "Phase I and pharmacokinetic study of intravenous irinotecan plus oral ciclosporin in patients with fuorouracil-refractory metastatic colon cancer." J Clin Oncol **21**(6): 1125-1132.

Chikakiyo, M., M. Shimada, et al. (2012). "Kampo medicine "Dai-kenchu-to" prevents CPT-11-induced small-intestinal injury in rats." Surg Today **42**(1): 60-67.

Chu, X. Y., Y. Kato, et al. (1997). "Multispecific organic anion transporter is responsible for the biliary excretion of the camptothecin derivative irinotecan and its metabolites in rats." J Pharmacol Exp Ther **281**(1): 304-314.

Chyka, P. A. (1995). "Multiple-dose activated charcoal and enhancement of systemic drug clearance: summary of studies in animals and human volunteers." J Toxicol Clin Toxicol **33**(5): 399-405.

Ciotti, M., N. Basu, et al. (1999). "Glucuronidation of 7-ethyl-10-hydroxycamptothecin (SN-38) by the human UDP-glucuronosyltransferases encoded at the UGT1 locus." Biochem Biophys Res Commun **260**(1): 199-202.

Cleary, J. M., K. T. Tanabe, et al. (2009). "Hepatic toxicities associated with the use of preoperative systemic therapy in patients with metastatic colorectal adenocarcinoma to the liver." Oncologist **14**(11): 1095-1105.

Combes, O., J. Barre, et al. (2000). "*In vitro* binding and partitioning of irinotecan (CPT-11) and its metabolite, SN-38, in human blood." Invest New Drugs **18**(1): 1-5.

Corona, G., C. Elia, et al. (2010). "Fast liquid chromatography-tandem mass spectrometry method for routine assessment of irinotecan metabolic phenotype." Ther Drug Monit **32**(5): 638-646.

Cunningham, D., S. Pyrhonen, et al. (1998). "Randomised trial of irinotecan plus supportive care versus supportive care alone after fluorouracil failure for patients with metastatic colorectal cancer." Lancet **352**(9138): 1413-1418.

Dai, J. Y., J. L. Yang, et al. (2008). "Transport and metabolism of flavonoids from Chinese herbal remedy Xiaochaihu- tang across human intestinal Caco-2 cell monolayers." Acta Pharmacol Sin **29**(9): 1086-1093.

Deng, G., R. C. Kurtz, et al. (2011). "A single arm phase II study of a Far-Eastern traditional herbal formulation (sho-sai-ko-to or xiao-chai-hu-tang) in chronic hepatitis C patients." J Ethnopharmacol **136**(1): 83-87.

Desai, A. A., H. L. Kindler, et al. (2005). "Modulation of irinotecan with cyclosporine: a phase II trial in advanced colorectal cancer." Cancer Chemother Pharmacol **56**(4): 421-426.

Dodds, H. M., M. C. Haaz, et al. (1998). "Identification of a new metabolite of CPT-11 (irinotecan): pharmacological properties and activation to SN-38." J Pharmacol Exp Ther **286**(1): 578-583.

Drengler, R. L., J. G. Kuhn, et al. (1999). "Phase I and pharmacokinetic trial of oral irinotecan administered daily for 5 days every 3 weeks in patients with solid tumors." J Clin Oncol **17**(2): 685-696.

Eisenberg, D. M., R. B. Davis, et al. (1998). "Trends in alternative medicine use in the United States, 1990-1997: results of a follow-up national survey." JAMA **280**(18): 1569-1575.

Fittkau, M., W. Voigt, et al. (2004). "Saccharic acid 1.4-lactone protects against CPT-11-induced mucosa damage in rats." J Cancer Res Clin Oncol **130**(7): 388-394.

Fukuoka, M., H. Niitani, et al. (1992). "A phase II study of CPT-11, a new derivative of camptothecin, for previously untreated non-small-cell lung cancer." J Clin Oncol **10**(1): 16-20.

Gagne, J. F., V. Montminy, et al. (2002). "Common human UGT1A polymorphisms and the altered metabolism of irinotecan active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38)." Mol Pharmacol **62**(3): 608-617.

Gandia, D., D. Abigerges, et al. (1993). "CPT-11-induced cholinergic effects in cancer patients." J Clin Oncol **11**(1): 196-197.

Gao, S., Z. Yang, et al. (2011). "Validated LC-MS/MS method for the determination of maackiain and its sulfate and glucuronide in blood: application to pharmacokinetic and disposition studies." J Pharm Biomed Anal **55**(2): 288-293.

Grottker, J., A. Rosenberger, et al. (2011). "Interaction of human multidrug and toxin extrusion 1 (MATE1) transporter with antineoplastic agents." Drug Metabol Drug Interact **26**(4): 181-189.

Gupta, E., T. M. Lestingi, et al. (1994). "Metabolic fate of irinotecan in humans: correlation of glucuronidation with diarrhea." Cancer Res **54**(14): 3723-3725.

Gupta, E., R. Mick, et al. (1997). "Pharmacokinetic and pharmacodynamic evaluation of the topoisomerase inhibitor irinotecan in cancer patients." J Clin Oncol **15**(4): 1502-1510.

Gupta, E., A. R. Safa, et al. (1996). "Pharmacokinetic modulation of irinotecan and metabolites by cyclosporin A." Cancer Res **56**(6): 1309-1314.

Haaz, M. C., L. Rivory, et al. (1998). "Metabolism of irinotecan (CPT-11) by human hepatic microsomes: participation of cytochrome P-450 3A and drug interactions." Cancer Res **58**(3): 468-472.

Hamaguchi, T., T. Doi, et al. (2010). "Phase I study of NK012, a novel SN-38-incorporating micellar nanoparticle, in adult patients with solid tumors." Clin Cancer Res **16**(20): 5058-5066.

Han, J. Y., H. S. Lim, et al. (2009). "Integrated pharmacogenetic prediction of irinotecan pharmacokinetics and toxicity in patients with advanced non-small cell lung cancer." Lung Cancer **63**(1): 115-120.

Hecht, J. R. (1998). "Gastrointestinal toxicity or irinotecan." Oncology (Williston Park) **12**(8 Suppl 6): 72-78.

Hecht, J. R., M. Pillai, et al. (2010). "A randomized, placebo-controlled phase ii study evaluating the reduction of neutropenia and febrile neutropenia in patients with colorectal cancer receiving pegfilgrastim with every-2-week chemotherapy." Clin Colorectal Cancer **9**(2): 95-101.

Herben, V. M., J. H. Schellens, et al. (1999). "Phase I and pharmacokinetic study of irinotecan administered as a low-dose, continuous intravenous infusion over 14 days in patients with malignant solid tumors." J Clin Oncol **17**(6): 1897-1905.

Hicks, L. D., J. L. Hyatt, et al. (2009). "Improved, selective, human intestinal carboxylesterase inhibitors designed to modulate 7-ethyl-10-[4-(1-piperidino)-1-

piperidino]carbonyloxycamptothecin (Irinotecan; CPT-11) toxicity." J Med Chem **52**(12): 3742-3752.

Hidaka, M., K. Yamasaki, et al. (2007). "Adsorption of irinotecan onto oral adsorbent AST-120 (Kremezin) for preventing delayed diarrhea." Cancer Chemother Pharmacol **59**(3): 321-328.

Hirose, K., C. Kozu, et al. (2012). "Correlation between plasma concentration ratios of SN-38 glucuronide and SN-38 and neutropenia induction in patients with colorectal cancer and wild-type UGT1A1 gene." Oncol Lett **3**(3): 694-698.

Horikawa, M., Y. Kato, et al. (2002). "Reduced gastrointestinal toxicity following inhibition of the biliary excretion of irinotecan and its metabolites by probenecid in rats." Pharm Res **19**(9): 1345-1353.

Hsiang, Y. H., R. Hertzberg, et al. (1985). "Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I." J Biol Chem **260**(27): 14873-14878.

Hu, M. and G. L. Amidon (1988). "Passive and carrier-mediated intestinal absorption components of captopril." J Pharm Sci **77**(12): 1007-1011.

Hu, M., K. Roland, et al. (1998). "Determination of absorption characteristics of AG337, a novel thymidylate synthase inhibitor, using a perfused rat intestinal model." J Pharm Sci **87**(7): 886-890.

Hu, Z. P., X. X. Yang, et al. (2006). "St. John's wort attenuates irinotecan-induced diarrhea via down-regulation of intestinal pro-inflammatory cytokines and inhibition of intestinal epithelial apoptosis." Toxicol Appl Pharmacol **216**(2): 225-237.

Ichikawa, W., K. Uehara, et al. (2015). "An internally and externally validated nomogram for predicting the risk of irinotecan-induced severe neutropenia in advanced colorectal cancer patients." Br J Cancer **112**(10): 1709-1716.

Innocenti, F., S. D. Undevia, et al. (2004). "A phase I trial of pharmacologic modulation of irinotecan with cyclosporine and phenobarbital." Clin Pharmacol Ther **76**(5): 490-502.

Itoh, T., S. Itagaki, et al. (2005). "Uptake of irinotecan metabolite SN-38 by the human intestinal cell line Caco-2." Cancer Chemother Pharmacol **55**(5): 420-424.

Itoh, T., I. Takemoto, et al. (2004). "Biliary excretion of irinotecan and its metabolites." J Pharm Pharm Sci **7**(1): 13-18.

Iyer, L., C. D. King, et al. (1998). "Genetic predisposition to the metabolism of irinotecan (CPT-11). Role of uridine diphosphate glucuronosyltransferase isoform 1A1 in the glucuronidation of its active metabolite (SN-38) in human liver microsomes." J Clin Invest **101**(4): 847-854.

Iyer, L., J. Ramirez, et al. (2002). "Biliary transport of irinotecan and metabolites in normal and P-glycoprotein-deficient mice." Cancer Chemother Pharmacol **49**(4): 336-341.

Javle, M. M., S. Cao, et al. (2007). "Celecoxib and mucosal protection: translation from an animal model to a phase I clinical trial of celecoxib, irinotecan, and 5-fluorouracil." Clin Cancer Res **13**(3): 965-971.

Kakumu, S., K. Yoshioka, et al. (1991). "Effects of TJ-9 Sho-saiko-to (kampo medicine) on interferon gamma and antibody production specific for hepatitis B virus antigen in patients with type B chronic hepatitis." Int J Immunopharmacol **13**(2-3): 141-146.

Kaneko, M., T. Kawakita, et al. (1994). "Augmentation of NK activity after oral administration of a traditional Chinese medicine, xiao-chai-hu-tang (shosaiko-to)." Immunopharmacol Immunotoxicol **16**(1): 41-53.

Kase, Y., T. Hayakawa, et al. (1997). "Relevance of irinotecan hydrochloride-induced diarrhea to the level of prostaglandin E2 and water absorption of large intestine in rats." Jpn J Pharmacol **75**(4): 399-405.

Katoh, M., Y. Yoshioka, et al. (2009). "Effects of Japanese herbal medicine, Kampo, on human UGT1A1 activity." Drug Metab Pharmacokinet **24**(3): 226-234.

Kehrer, D. F., A. Sparreboom, et al. (2001). "Modulation of irinotecan-induced diarrhea by cotreatment with neomycin in cancer patients." Clin Cancer Res **7**(5): 1136-1141.

Khanna, R., C. L. Morton, et al. (2000). "Proficient metabolism of irinotecan by a human intestinal carboxylesterase." Cancer Res **60**(17): 4725-4728.

Kim, T. W. and F. Innocenti (2007). "Insights, challenges, and future directions in irinogenetics." Ther Drug Monit **29**(3): 265-270.

Kojima, A., N. R. Hackett, et al. (1998). "In vivo human carboxylesterase cDNA gene transfer to activate the prodrug CPT-11 for local treatment of solid tumors." J Clin Invest **101**(8): 1789-1796.

Kummar, S., M. S. Copur, et al. (2011). "A phase I study of the chinese herbal medicine PHY906 as a modulator of irinotecan-based chemotherapy in patients with advanced colorectal cancer." Clin Colorectal Cancer **10**(2): 85-96.

Kurita, A., S. Kado, et al. (2000). "Modified irinotecan hydrochloride (CPT-11) administration schedule improves induction of delayed-onset diarrhea in rats." Cancer Chemother Pharmacol **46**(3): 211-220.

Kurita, A., S. Kado, et al. (2003). "Alleviation of side effects induced by irinotecan hydrochloride (CPT-11) in rats by intravenous infusion." Cancer Chemother Pharmacol **52**(5): 349-360.

Kurita, A., S. Kado, et al. (2011). "Streptomycin alleviates irinotecan-induced delayed-onset diarrhea in rats by a mechanism other than inhibition of beta-glucuronidase activity in intestinal lumen." Cancer Chemother Pharmacol **67**(1): 201-213.

Kurzrock, R., S. Goel, et al. (2012). "Safety, pharmacokinetics, and activity of EZN-2208, a novel conjugate of polyethylene glycol and SN38, in patients with advanced malignancies." Cancer **118**(24): 6144-6151.

Lam, W., S. Bussom, et al. (2010). "The four-herb Chinese medicine PHY906 reduces chemotherapy-induced gastrointestinal toxicity." Sci Transl Med **2**(45): 45ra59.

Lenfers, B. H., T. M. Loeffler, et al. (1999). "Substantial activity of budesonide in patients with irinotecan (CPT-11) and 5-fluorouracil induced diarrhea and failure of loperamide treatment." Ann Oncol **10**(10): 1251-1253.

Liu, S. H. and Y. C. Cheng (2012). "Old formula, new Rx: the journey of PHY906 as cancer adjuvant therapy." J Ethnopharmacol **140**(3): 614-623.

Liu, Y. and M. Hu (2002). "Absorption and metabolism of flavonoids in the caco-2 cell culture model and a perused rat intestinal model." Drug Metab Dispos **30**(4): 370-377.

Lokiec, F., B. M. du Sorbier, et al. (1996). "Irinotecan (CPT-11) metabolites in human bile and urine." Clin Cancer Res **2**(12): 1943-1949.

Luo, F. R., P. V. Paranjpe, et al. (2002). "Intestinal transport of irinotecan in Caco-2 cells and MDCK II cells overexpressing efflux transporters Pgp, cMOAT, and MRP1." Drug Metab Dispos **30**(7): 763-770.

Ma, M. K. and H. L. McLeod (2003). "Lessons learned from the irinotecan metabolic pathway." Curr Med Chem **10**(1): 41-49.

Mathijssen, R. H., R. J. van Alphen, et al. (2001). "Clinical pharmacokinetics and metabolism of irinotecan (CPT-11)." Clin Cancer Res **7**(8): 2182-2194.

Matuszewski, B. K., M. L. Constanzer, et al. (2003). "Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS." Anal Chem **75**(13): 3019-3030.

Merrouche, Y., J. M. Extra, et al. (1997). "High dose-intensity of irinotecan administered every 3 weeks in advanced cancer patients: a feasibility study." J Clin Oncol **15**(3): 1080-1086.

Nagar, S. and R. L. Blanchard (2006). "Pharmacogenetics of uridine diphosphoglucuronosyltransferase (UGT) 1A family members and its role in patient response to irinotecan." Drug Metab Rev **38**(3): 393-409.

Narita, M., E. Nagai, et al. (1993). "Inhibition of beta-glucuronidase by natural glucuronides of kampo medicines using glucuronide of SN-38 (7-ethyl-10-hydroxycamptothecin) as a substrate." Xenobiotica **23**(1): 5-10.

Nose, M., M. Tamura, et al. (2003). "Sho-saiko-to and Saiko-keisi-to, the traditional Chinese and Japanese herbal medicines, altered hepatic drug-metabolizing enzymes in mice and rats when administered orally for a long time." J Pharm Pharmacol **55**(10): 1419-1426.

Ohno, R., K. Okada, et al. (1990). "An early phase II study of CPT-11: a new derivative of camptothecin, for the treatment of leukemia and lymphoma." J Clin Oncol **8**(11): 1907-1912.

Oka, H., S. Yamamoto, et al. (1995). "Prospective study of chemoprevention of hepatocellular carcinoma with Sho-saiko-to (TJ-9)." Cancer **76**(5): 743-749.

Park, D. J., J. H. Won, et al. (2014). "Determination of irinotecan and its metabolite SN-38 in rabbit plasma and tumors using a validated method of tandem mass spectrometry coupled with liquid chromatography." J Chromatogr B Analyt Technol Biomed Life Sci **962**: 147-152.

Prados, M. D., W. K. Yung, et al. (2004). "Phase 1 trial of irinotecan (CPT-11) in patients with recurrent malignant glioma: a North American Brain Tumor Consortium study." Neuro Oncol **6**(1): 44-54.

Redinbo, M. R., L. Stewart, et al. (1998). "Crystal structures of human topoisomerase I in covalent and noncovalent complexes with DNA." Science **279**(5356): 1504-1513.

Rosen, L. S. (1998). "Irinotecan in lymphoma, leukemia, and breast, pancreatic, ovarian, and small-cell lung cancers." Oncology (Williston Park) **12**(8 Suppl 6): 103-109.

Rosenoff, S. H. (2004). "Octreotide LAR resolves severe chemotherapy-induced diarrhoea (CID) and allows continuation of full-dose therapy." Eur J Cancer Care (Engl) **13**(4): 380-383.

Rothenberg, M. L. (1998). "Efficacy and toxicity of irinotecan in patients with colorectal cancer." Semin Oncol **25**(5 Suppl 11): 39-46.

Rothenberg, M. L., J. G. Kuhn, et al. (1993). "Phase I and pharmacokinetic trial of weekly CPT-11." J Clin Oncol **11**(11): 2194-2204.

Rothenberg, M. L., J. G. Kuhn, et al. (1998). "Alternative dosing schedules for irinotecan." Oncology (Williston Park) **12**(8 Suppl 6): 68-71.

Rothenberg, M. L., N. J. Meropol, et al. (2001). "Mortality associated with irinotecan plus bolus fluorouracil/leucovorin: summary findings of an independent panel." J Clin Oncol **19**(18): 3801-3807.

Rougier, P., R. Bugat, et al. (1997). "Phase II study of irinotecan in the treatment of advanced colorectal cancer in chemotherapy-naive patients and patients pretreated with fluorouracil-based chemotherapy." J Clin Oncol **15**(1): 251-260.

Rowinsky, E. K., L. B. Grochow, et al. (1994). "Phase I and pharmacological study of the novel topoisomerase I inhibitor 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) administered as a ninety-minute infusion every 3 weeks." Cancer Res **54**(2): 427-436.

Ruppin, H. (1987). "Review: loperamide--a potent antidiarrhoeal drug with actions along the alimentary tract." Aliment Pharmacol Ther **1**(3): 179-190.

Sakai, H., M. Diener, et al. (1995). "Eicosanoid-mediated Cl- secretion induced by the antitumor drug, irinotecan (CPT-11), in the rat colon." Naunyn Schmiedebergs Arch Pharmacol **351**(3): 309-314.

Sakai, H., T. Sato, et al. (1997). "Thromboxane A2, released by the anti-tumour drug irinotecan, is a novel stimulator of Cl- secretion in isolated rat colon." J Physiol **505 (Pt 1)**: 133-144.

Sakaida, I., Y. Matsumura, et al. (1998). "Herbal medicine Sho-saiko-to (TJ-9) prevents liver fibrosis and enzyme-altered lesions in rat liver cirrhosis induced by a choline-deficient L-amino acid-defined diet." J Hepatol **28**(2): 298-306.

Saltz, L. B., J. V. Cox, et al. (2000). "Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group." N Engl J Med **343**(13): 905-914.

Sanghani, S. P., S. K. Quinney, et al. (2004). "Hydrolysis of irinotecan and its oxidative metabolites, 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin and 7-ethyl-10-[4-(1-piperidino)-1-amino]-carbonyloxycamptothecin, by human carboxylesterases CES1A1, CES2, and a newly expressed carboxylesterase isoenzyme, CES3." Drug Metab Dispos **32**(5): 505-511.

Sano, K., M. Yoshikawa, et al. (2003). "Simple non-ion-paired high-performance liquid chromatographic method for simultaneous quantitation of carboxylate and lactone forms of 14 new camptothecin derivatives." J Chromatogr B Analyt Technol Biomed Life Sci **795**(1): 25-34.

Santos, A., S. Zanetta, et al. (2000). "Metabolism of irinotecan (CPT-11) by CYP3A4 and CYP3A5 in humans." Clin Cancer Res **6**(5): 2012-2020.

Sawada, S., S. Okajima, et al. (1991). "Synthesis and antitumor activity of 20(S)-camptothecin derivatives: carbamate-linked, water-soluble derivatives of 7-ethyl-10-hydroxycamptothecin." Chem Pharm Bull (Tokyo) **39**(6): 1446-1450.

Schoemaker, N. E., I. E. Kuppens, et al. (2005). "Phase I study of an oral formulation of irinotecan administered daily for 14 days every 3 weeks in patients with advanced solid tumours." Cancer Chemother Pharmacol **55**(3): 263-270.

Sergio, G. C., G. M. Felix, et al. (2008). "Activated charcoal to prevent irinotecan-induced diarrhea in children." Pediatr Blood Cancer **51**(1): 49-52.

Shao, R. G., C. X. Cao, et al. (1999). "Replication-mediated DNA damage by camptothecin induces phosphorylation of RPA by DNA-dependent protein kinase and dissociates RPA:DNA-PK complexes." EMBO J **18**(5): 1397-1406.

Shimada, Y., M. Yoshino, et al. (1993). "Phase II study of CPT-11, a new camptothecin derivative, in metastatic colorectal cancer. CPT-11 Gastrointestinal Cancer Study Group." J Clin Oncol **11**(5): 909-913.

Slatter, J. G., L. J. Schaaf, et al. (2000). "Pharmacokinetics, metabolism, and excretion of irinotecan (CPT-11) following I.V. infusion of [(14)C]CPT-11 in cancer patients." Drug Metab Dispos **28**(4): 423-433.

Slichenmyer, W. J., E. K. Rowinsky, et al. (1993). "The current status of camptothecin analogues as antitumor agents." J Natl Cancer Inst **85**(4): 271-291.

Smith, N. F., W. D. Figg, et al. (2006). "Pharmacogenetics of irinotecan metabolism and transport: an update." Toxicol In Vitro **20**(2): 163-175.

Song, K. H., Y. H. Kim, et al. (2014). "Sho-saiko-to, a traditional herbal medicine, regulates gene expression and biological function by way of microRNAs in primary mouse hepatocytes." BMC Complement Altern Med **14**: 14.

Sparreboom, A., M. J. de Jonge, et al. (1998). "Irinotecan (CPT-11) metabolism and disposition in cancer patients." Clin Cancer Res **4**(11): 2747-2754.

Stein, A., W. Voigt, et al. (2010). "Chemotherapy-induced diarrhea: pathophysiology, frequency and guideline-based management." Ther Adv Med Oncol **2**(1): 51-63.

Stewart, C. F., M. Leggas, et al. (2004). "Gefitinib enhances the antitumor activity and oral bioavailability of irinotecan in mice." Cancer Res **64**(20): 7491-7499.

Stewart, L., M. R. Redinbo, et al. (1998). "A model for the mechanism of human topoisomerase I." Science **279**(5356): 1534-1541.

Sugiyama, Y., Y. Kato, et al. (1998). "Multiplicity of biliary excretion mechanisms for the camptothecin derivative irinotecan (CPT-11), its metabolite SN-38, and its glucuronide: role of canalicular multispecific organic anion transporter and P-glycoprotein." Cancer Chemother Pharmacol **42 Suppl**: S44-49.

Takahashi, K., E. Uejima, et al. (2003). "*In vitro* inhibitory effects of Kampo medicines on metabolic reactions catalyzed by human liver microsomes." J Clin Pharm Ther **28**(4): 319-327.

Takahashi, Y., Y. Soejima, et al. (2014). "Inhibitory effects of Japanese herbal medicines sho-saiko-to and juzen-taiho-to on nonalcoholic steatohepatitis in mice." PLoS One **9**(1): e87279.

Takasuna, K., T. Hagiwara, et al. (1996). "Involvement of beta-glucuronidase in intestinal microflora in the intestinal toxicity of the antitumor camptothecin derivative irinotecan hydrochloride (CPT-11) in rats." Cancer Res **56**(16): 3752-3757.

Takasuna, K., T. Hagiwara, et al. (1998). "Inhibition of intestinal microflora beta-glucuronidase modifies the distribution of the active metabolite of the antitumor agent, irinotecan hydrochloride (CPT-11) in rats." Cancer Chemother Pharmacol **42**(4): 280-286.

Takasuna, K., Y. Kasai, et al. (1995). "Protective effects of kampo medicines and baicalin against intestinal toxicity of a new anticancer camptothecin derivative, irinotecan hydrochloride (CPT-11), in rats." Jpn J Cancer Res **86**(10): 978-984.

Takeda, Y., K. Kobayashi, et al. (2001). "Prevention of irinotecan (CPT-11)-induced diarrhea by oral alkalization combined with control of defecation in cancer patients." Int J Cancer **92**(2): 269-275.

Takimoto, C. H., G. Morrison, et al. (2000). "Phase I and pharmacologic study of irinotecan administered as a 96-hour infusion weekly to adult cancer patients." J Clin Oncol **18**(3): 659-667.

Tamura, T., K. Yasutake, et al. (2004). "Prevention of irinotecan-induced diarrhea by oral sodium bicarbonate and influence on pharmacokinetics." Oncology **67**(5-6): 327-337.

Tobin, P. J., P. Beale, et al. (2006). "A pilot study on the safety of combining chrysin, a non-absorbable inducer of UGT1A1, and irinotecan (CPT-11) to treat metastatic colorectal cancer." Cancer Chemother Pharmacol **57**(3): 309-316.

Tournigand, C., T. Andre, et al. (2004). "FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study." J Clin Oncol **22**(2): 229-237.

Trifan, O. C., W. F. Durham, et al. (2002). "Cyclooxygenase-2 inhibition with celecoxib enhances antitumor efficacy and reduces diarrhea side effect of CPT-11." Cancer Res **62**(20): 5778-5784.

Tukey, R. H. and C. P. Strassburg (2000). "Human UDP-glucuronosyltransferases: metabolism, expression, and disease." Annu Rev Pharmacol Toxicol **40**: 581-616.

Valenti Moreno, V., J. Brunet Vidal, et al. (2006). "Prevention of irinotecan associated diarrhea by intestinal alkalization. A pilot study in gastrointestinal cancer patients." Clin Transl Oncol **8**(3): 208-212.

Vasudev, N. S., S. Jagdev, et al. (2005). "Intravenous irinotecan plus oral ciclosporin." Clin Oncol (R Coll Radiol) **17**(8): 646-649.

Wall M. E., W. M. C., Cook C. E., Palmer K. H., McPhail A. T., Sim G. A. (1966). "Plant antitumor agents. I. The isolation and structure of camptothecine, a novel alkaloidal leukemia and tumor inhibitor from Camptotheca acuminata." Journal of the American Chemical Society **88**(16): 3888-3890.

Wall M. E., W. M. C., Cook C. E., Palmer K. H., McPhail A. T., Sim G. A. (1966). "Plant antitumor agents. I. The isolation and structure of camptothecine, a novel alkaloidal leukemia and tumor inhibitor from Camptotheca acuminata." Journal of the American Chemical Society **88**(16): 3888-3890.

Wallace, B. D., H. Wang, et al. (2010). "Alleviating cancer drug toxicity by inhibiting a bacterial enzyme." Science **330**(6005): 831-835.

Wittwer, M. B., A. A. Zur, et al. (2013). "Discovery of potent, selective multidrug and toxin extrusion transporter 1 (MATE1, SLC47A1) inhibitors through prescription drug profiling and computational modeling." J Med Chem **56**(3): 781-795.

Wong, R., C. M. Sagar, et al. (2001). "Integration of Chinese medicine into supportive cancer care: a modern role for an ancient tradition." Cancer Treat Rev **27**(4): 235-246.

Xie, R., R. H. Mathijssen, et al. (2002). "Clinical pharmacokinetics of irinotecan and its metabolites: a population analysis." J Clin Oncol **20**(15): 3293-3301.

Yamashiki, M., A. Nishimura, et al. (1997). "Effects of the Japanese herbal medicine "Sho-saiko-to" (TJ-9) on *in vitro* interleukin-10 production by peripheral blood mononuclear cells of patients with chronic hepatitis C." Hepatology **25**(6): 1390-1397.

Yang, C. J., J. K. Horton, et al. (1995). "Cross-resistance to camptothecin analogues in a mitoxantrone-resistant human breast carcinoma cell line is not due to DNA topoisomerase I alterations." Cancer Res **55**(18): 4004-4009.

Yang, X., Z. Hu, et al. (2005). "Novel agents that potentially inhibit irinotecan-induced diarrhea." Curr Med Chem **12**(11): 1343-1358.

Yang, Z., S. Gao, et al. (2010). "Biopharmaceutical and pharmacokinetic characterization of matrine as determined by a sensitive and robust UPLC-MS/MS method." J Pharm Biomed Anal **51**(5): 1120-1127.

Ychou, M., J. Y. Douillard, et al. (2000). "Randomized comparison of prophylactic antidiarrheal treatment versus no prophylactic antidiarrheal treatment in patients receiving CPT-11 (irinotecan) for advanced 5-FU-resistant colorectal cancer: an open-label multicenter phase II study." Am J Clin Oncol **23**(2): 143-148.

Yokoi, T., M. Narita, et al. (1995). "Inhibition of UDP-glucuronosyltransferase by aglycons of natural glucuronides in kampo medicines using SN-38 as a substrate." Jpn J Cancer Res **86**(10): 985-989.

Yoon, K. J., J. L. Hyatt, et al. (2004). "Characterization of inhibitors of specific carboxylesterases: development of carboxylesterase inhibitors for translational application." Mol Cancer Ther **3**(8): 903-909.

Zampa, G., E. Magnolfi, et al. (2000). "Premedication for irinotecan." J Clin Oncol **18**(1): 237.

Zhang, J. A., T. Xuan, et al. (2004). "Development and characterization of a novel liposome-based formulation of SN-38." Int J Pharm **270**(1-2): 93-107.

Zhang, W., M. W. Saif, et al. (2010). "Identification of chemicals and their metabolites from PHY906, a Chinese medicine formulation, in the plasma of a patient treated with irinotecan and PHY906 using liquid chromatography/tandem mass spectrometry (LC/MS/MS)." J Chromatogr A **1217**(37): 5785-5793.

Zhang, X. X., W. S. Pan, et al. (2008). "Effects of breast cancer resistance protein inhibitors and pharmaceutical excipients on decreasing gastrointestinal toxicity of camptothecin analogs." Acta Pharmacol Sin **29**(11): 1391-1398.